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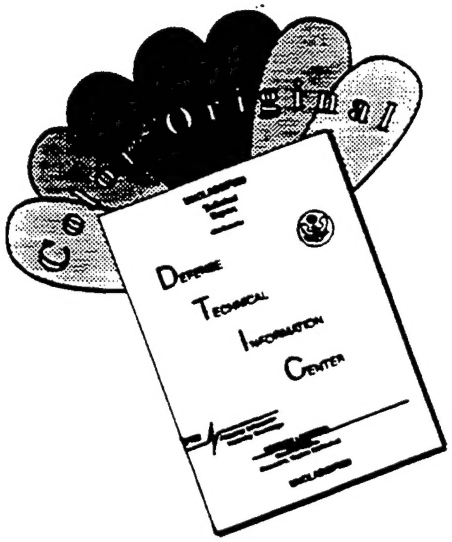

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This research is aimed at elucidating why breast cancer cells become resistant to antiestrogen treatment. Antiestrogens are used widely in the treatment of breast cancer, and although almost 50% of breast cancer patients benefit substantially from treatment with tamoxifen, many of these women eventually suffer relapse because some of the breast cancer cells become resistant to tamoxifen. We find that cyclic AMP stimulates the agonist activity of tamoxifen-like antiestrogens and reduces the ability of these antiestrogens to suppress estrogen-stimulated activity. In addition, estrogens and antiestrogens increase intracellular cAMP levels, which make tamoxifen more estrogenic and compromise its suppressive ability. We have developed several model breast cancer cell systems that differ in their sensitivity/resistance to antiestrogens and have characterized alterations in their proliferation, their production of and responsiveness to the transforming growth factors α and β, their intracellular cAMP levels, and their production of other estrogen-responsive proteins. We are using these breast cancer cells to study the role of intracellular cAMP in augmentation of antiestrogen agonist activity and in compromising the effectiveness of antiestrogens as estrogen antagonists, which may form the basis for tamoxifen resistance. Understanding the basis for the development of tamoxifen resistance will be important in developing more effective strategies for the successful long-term treatment of hormone-responsive breast cancer.

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Rita S. Kadyellby 10/22/96

PI - Signature Date

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INTRODUCTION: Nature of the Problem, Background, Purpose of the Present Work, and Methods of Approach

This research is aimed at elucidating why breast cancer cells become resistant to antiestrogen treatment. Antiestrogens are used widely in the treatment of breast cancer, but development of resistance and patient relapse is a significant problem. The antiestrogen tamoxifen is the most widely prescribed drug for breast cancer treatment and it is usually considered the treatment of choice for the endocrine therapy of breast cancer because of its effectiveness, ease of use, and minimal side effects. It may also be of benefit in preventing the development of breast cancer in women at high risk for the disease, a hypothesis being currently tested in a major NCI-funded clinical trial. Although almost one-half of breast cancer patients benefit substantially from treatment with tamoxifen, many of these women eventually suffer relapse because some of the breast cancer cells have become resistant to tamoxifen. This resistance to tamoxifen presents a major impediment to the long-term effectiveness of such treatments. Our research is aimed at understanding and elucidating why breast cancer cells become resistant to antiestrogen treatment. In these studies we are using several model human breast cancer cell systems that differ in their sensitivity and resistance to tamoxifen, and we are investigating a novel mechanism and hypothesis that may explain antiestrogen resistance, namely the stimulation of adenylate cyclase by antiestrogens with increases in intracellular cAMP, augmentation of antiestrogen agonist character, and reduced effectiveness of antiestrogens as estrogen antagonists.

Clinical experience has shown that hormonal resistance is often reversible, suggesting a cellular adaptation mechanism, rather than a genetic alteration in many breast cancers. This also seems to be the case in the tamoxifen-resistant human breast cancer cells (denoted MCF/TOT) we have developed (M. Herman and B. Katzenellenbogen, publication #8), and which are described in the section below entitled "Body". For example, patients that become resistant to tamoxifen often respond immediately to treatments with high dose estrogen or return to a state of tamoxifen responsiveness after a period of alternative therapy. Therefore, any mechanism that would explain tamoxifen resistance in these patients would have to involve mechanisms that would be reversible or adaptational, in contrast to other mechanisms for tamoxifen resistance that might involve mutations in the estrogen receptor or other critical transcription factor or growth factor genes. Therefore, we have been further investigating our observations regarding a two-way link between estrogen receptors and cAMP which would be consistent with a reversible and adaptational mechanism of antiestrogen resistance. Our observations that estrogens as well as antiestrogens are able to increase cAMP in breast cancer cells, and that cAMP increases the stimulatory effects of tamoxifen-like antiestrogens, could result in a feed-forward cascade that could result in the total compromising of the tumor growth suppressing activities of antiestrogens.

It is noteworthy the cAMP levels are significantly higher in breast tumors than in normal breast tissue and that elevated concentrations of cAMP binding proteins are associated with early disease recurrence and poor survival rates. Interestingly, as well, cAMP is both a mitogenic and a morphogenic factor in mammary cells and it has been shown to enhance the mitogenic activity of several growth factors. Therefore, our overall goal in these studies is to develop an understanding of the basis for the development of tamoxifen resistance in breast cancer. Understanding the basis for the development of tamoxifen resistance would be an important first step in developing more effective strategies for the successful long-term treatment of hormone-responsive breast cancer. In addition, this research should allow us to develop more effective therapies for antiestrogen-sensitive and antiestrogen-resistant breast cancers and should enable the use of antiestrogens to be approached most sensibly and effectively in the clinic.

BODY: Experimental Methods Used, Results Obtained and the Relationship of Our Results to the Goals of the Research

In this past year, we have made good progress on the Specific Aims. As detailed below, we have completed Statement of Work Tasks 1 and 2, have begun on validation of some parameters related to Task 3, and we have already made a few mutant estrogen receptors that we plan to study in Task 4 to identify sites of estrogen receptor phosphorylation regulated by the cAMP pathway and kinases activated by cAMP elevation in breast cancer cells.

Since we have shown that estrogens and antiestrogens increase cAMP within breast cancer cells (Aronica, S. M., Kraus, W. L., and Katzenellenbogen B. S., Proc. Natl. Acad. Sci. USA 91: 8517-8521, 1994), and cAMP alters the agonist/antagonist balance of tamoxifen-like antiestrogens (Fujimoto, N., and Katzenellenbogen, B. S. Molec. Endocrinol. 8: 296-304, 1994), the increase in cAMP may result in a reduction in the tumor growth-suppressing activity of tamoxifen, a change that may underlie the development of tamoxifen resistance in some breast cancer patients. To examine this hypothesis in detail, we have isolated and characterized antiestrogen-resistant MCF-7 human breast cancer sublines that we have selected and cloned, and we have determined their responses to antiestrogens and cAMP in terms of cell proliferation and growth factor production, and the responses of other genes normally estrogen regulated, such as progesterone receptor and pS2 (Herman and Katzenellenbogen, publication #8, and Nicholson et al, publication #1 and Nicholson et al, publication #4, and Ince et al, publication #3, and Katzenellenbogen et al, publication #9). These studies have directly addressed the Statement of Work Task 1, points a, b, c and d.

For these studies, we cultured MCF-7 breast cancer cells long-term (longer than 1 year) in the presence of the antiestrogen *trans*-hydroxy-tamoxifen (TOT) to generate a subline refractory to the growth-suppressive effects of TOT. This subline

(designated MCF/TOT) showed growth stimulation, rather than inhibition, with TOT and diminished growth stimulation with estradiol (E_2), yet remained as sensitive as the parental cells to growth suppression by another antiestrogen, ICI 164,384. Estrogen receptor (ER) levels were maintained at 40% that in parent MCF-7 cells, but MCF/TOT cells failed to show an increase in progesterone receptor content in response to E_2 or TOT treatment. In contrast, the MCF/TOT subline behaved like parental cells in terms of E_2 and TOT regulation of ER and pS2 expression and transactivation of a transiently transfected estrogen-responsive gene construct. DNA sequencing of the hormone binding domain of the ER from both MCF-7 and MCF/TOT cells confirmed the presence of wild-type ER and exon 5 and exon 7 deletion splice variants, but showed no point mutations. Compared to the parental cells, the MCF/TOT subline showed reduced sensitivity to the growth-suppressive effects of retinoic acid and complete resistance to exogenous TGF-β1. The altered growth responsiveness of MCF/TOT cells to TOT and TGF-β1 was partially to fully reversible following TOT withdrawal for 16 weeks. Our findings underscore the fact that antiestrogen resistance is response-specific; that loss of growth suppression by TOT appears to be due to the acquisition of weak growth stimulation; and that resistance to TOT does not mean global resistance to other more pure antiestrogens such as ICI 164,384, implying that these antiestrogens must act by somewhat different mechanisms. The association of reduced retinoic acid responsiveness and insensitivity to exogenous TGF-β with antiestrogen growth-resistance in these cells supports the increasing evidence for interrelationships among cell regulatory pathways utilized by these three growth-suppressive agents in breast cancer cells. In addition, our findings indicate that one mechanism of antiestrogen resistance, as seen in MCF/TOT cells, may involve alterations in growth factor and other hormonal pathways that affect the ER response pathway.

Since these MCF/TOT cells, resistant to the growth suppressive effects of antiestrogens or TGF-β continue to express TGF-β type I and II receptors of the correct size and in amounts equal to those observed in the parental cells, their lack of inhibition by the high levels of TGF-β1 either being made by the cells or added by us to their culture media suggest a lesion after receptor binding, i.e. at some point in the TGF-β intracellular signalling pathway. We have also used several MCF-7 cell clones with altered antiestrogen sensitivity to investigate the response to cAMP and antiestrogen as monitored by proliferation rates, colony formation ability and changes in regulation of several growth-related genes (TGF-β, TGF-α, pS2, and TGF-α/EGF receptor), (Publications # 1, 4, and 8). In addition, we have studied the regulation of the progesterone receptor in tamoxifen- and estrogen-sensitive and tamoxifen- and estrogen-resistant breast cancer cells, since the progesterone receptor is often used as an end-point or marker of hormone sensitivity and responsiveness. By monitoring progesterone receptor content in the cells, using several different progesterone receptor-specific antibodies, we have observed that the progesterone

receptor B/A ratio is higher with trans-hydroxytamoxifen versus estrogen treatment of cells (a variety of different estrogens were tested) and progesterone receptors were further increased by treatment of cells with 8-Br-cAMP and trans-hydroxytamoxifen.

We have monitored basal and stimulated levels of cAMP in parental MCF-7 cells and in our MCF/TOT (tamoxifen stimulated) MCF-7 cells and in estrogen receptor negative MDA-MB-231 breast cancer cells which are unresponsive to estrogen and antiestrogen. We have found that the antiestrogen-stimulated MCF-7 cells and the antiestrogen-unresponsive 231 cells showed 3-5 times higher intracellular cAMP levels than were observed in the parental MCF-7 cells. We observed no stimulation of cAMP levels by estrogen or antiestrogen treatment of 231 cells, while we observed only a 1.5-fold change in cAMP in the MCF/TOT cells and we observed a 3-4 fold increase in cAMP in the parental MCF-7 cells. Thus, hormone resistant and antiestrogen stimulated cells interestingly had elevated basal levels of cAMP, an observation we also made in breast cancer cells studied under Task 2, that were kindly provided by Dr. Fran Kern of the Lombardi Cancer Research Center at Georgetown University in Washington D. C.

Under Task 2, we have worked towards the identification of endogenous and exogenous agents and factors that result in elevation of cAMP levels in breast cancer cells. We have investigated the correlation between antiestrogen growth responsiveness/resistance and cellular cAMP levels and adenylate cyclase activities. Using 5 breast cancer cells lines (MCF-7 wild type versus MCF-7 tamoxifen stimulated, and 3 MCF-7 cell lines that are resistant to antiestrogen (MCF-7-v-Ha-ras, MCF-7-FGF1 and MCF-7-FGF4, which stably overexpress ras, FGF-1, or FGF-4, respectively, kindly provided to us by Dr. Fran Kern, we have observed that the overexpressing ras and FGF cells show basal cAMP levels 2.5-3.5 x higher than wild type MCF-7 cells. Values obtained were as follows (mean ± S. D. : wild type MCF-7 cells, 35 ± 10; MCF-7 ras, 121 ± 9; MCF-7 FGF-1, 86 ± 2; MCF-7 FGF-4, 103 ± 9. Interestingly, these latter three cell types, which proliferate rapidly and do not have their rate of proliferation influenced by estrogen or antiestrogen, likewise did not have their intracellular cAMP levels influenced by estrogen or antiestrogen treatment. Thus, elevated levels of cellular cAMP appear to correlate with altered growth responsiveness/resistance and with an estrogen and antiestrogen growth-autonomous state.

We also asked whether estradiol would affect intracellular cAMP in human endometrial cancer Ishikawa cells. These cells contain estrogen receptor and were of interest because tamoxifen is known to be quite agonistic (i.e. stimulatory) in endometrial cells, and in fact, a major concern in the Tamoxifen Prevention Trial in women has involved stimulation of the uterus by tamoxifen. We observed in these cells, basal and estrogen-stimulated and isobutyl methyl xanthine (IBMX)/cholera toxin-stimulated levels of cAMP similar in magnitude to those observed in the MCF-7 wild type breast cancer cells, namely an approximately 20-fold increase in

response to IBMX and cholera toxin and an approximately 3-6 fold increase in response to estradiol. Thus, these uterine cells did not show a response to estrogen or to tamoxifen substantially different in magnitude from that observed with MCF-7 breast cancer cells.

Since antiestrogens such as tamoxifen can have partial estrogen-like activity in some cell types, and studies have implied that this stimulation is dependent on the amino-terminal activation function-1-containing region of the receptor, we studied this region of the receptor in detail (McInerney, EM and Katzenellenbogen BS, publication #13). In our investigations on the A/B domain of the estrogen receptor and its role in the transcriptional activity of the estrogen receptor elicited by estrogens and some antiestrogens, we have found that different regions within this domain are required for transcriptional stimulation by estrogen versus antiestrogen. We demonstrated that a specific 24-amino acid region of activation function-1 of the human estrogen receptor is necessary for agonism by trans-hydroxytamoxifen and other partial agonist/antagonist antiestrogens, but is not required for estradiol-dependent transactivation. As a consequence, the activity of estradiol and the estrogen agonist/antagonist character of trans-hydroxytamoxifen depended markedly, but not always concordantly, on the sequences present within the A/B domain in the receptor. Our studies show that hormone-dependent transcription utilizes a broad range of sequences within the amino terminal A/B domain and suggest that differences in the agonist/antagonist character of antiestrogens observed in cells could be due to altered levels of specific factors that interact with these regions of the receptor protein.

During our work this past year, a publication appeared in which a group of Italian researchers reported that sex steroid binding globulin (SSBG) was necessary in the stimulation of cAMP by estrogen in breast cancer cells (F. Fissore et al., Steroids 59:661-667, 1994). Because we felt it was essential for us to determine if this was important in our work related to Task 1c and 1d, and in the identification of membrane sites (related to Task 3), we purchased SSBG from two different sources, namely Calbiochem and Scripps Laboratories, both SSBG preparations in highly purified form. We followed the Fissore protocol as closely as possible and also did several variations. Thus, we utilized 1nM and 3nM SSBG concentrations with cells in serum-free medium, and in 0.5% and 5% serum, and with cells in serum-free medium containing insulin, transferin and selenium. We also tested several different concentrations of estradiol, namely 10^{-8}, 10^{-9} and 10^{-10} M. In no case, did we observe a stimulatory (nor a suppressive) effect of SSBG on the cAMP response to hormone. Thus, despite several months of experiments, we were not able to confirm that sex steroid binding globulin was necessary for the stimulation of cAMP by hormone in our breast cancer cells. We therefore have ruled this out as a likely important factor in our studies in Tasks 1 and 3.

We have begun some work on the studies under Task 4, although many further studies are necessary and will continue over the next two years. The estrogen receptor contains two potential cAMP-dependent protein kinase sites at serine 236 in the DNA binding domain and serine 302 at the very start of the hormone binding domain. We therefore have changed these serines to alanines by site-directed oligonucleotide mutagenesis of the estrogen receptor cDNA. The change from serine to alanine would thus eliminate the possibility of phosphorylation at these sites. We will soon be testing the response of these mutants to cAMP and estrogen and antiestrogen in order to identify sites of phosphorylation that may be associated with the alteration in tamoxifen agonist character in the presence of cAMP.

Response to Technical Issue in Year 1 Report

(In our Year 1 Report, and in several published papers, we have observed that cAMP enhances the stimulatory activity of tamoxifen-like antiestrogens, but not the activity of ICI 164,384-type antiestrogens. The question was raised by reviewers of our Year 1 Report under "technical issues", whether these results might reflect differences in purity between the two drugs rather than true differences in the mechanisms of actions of these two different categories of antiestrogens. Our work and the work of others indicates that the tamoxifen-like antiestrogens and the ICI 164,384-type-antiestrogens work through the estrogen receptor of breast cancer cells but via quite different mechanisms. While tamoxifen-type antiestrogens are able to activate the activation function-1 of the receptor, the ICI-type antiestrogens fail to activate activation function-1 and instead accelerate degradation of the estrogen receptor reducing receptor content in the cells, and therefore preventing response to antiestrogen. Thus, the ability of ICI 164,384-like antiestrogens to reverse the stimulatory effect of tamoxifen-like antiestrogens, and the inability of estrogen receptor-ICI antiestrogen complexes to be stimulated by elevated intracellular cAMP, we believe, reflects a true difference in the mechanism of action of these compounds and does not relate at all to purity of the compounds, which are both very pure as we use them.)

CONCLUSIONS: Implications of Our Research Findings,and Future Work to be Undertaken

The results of our studies indicate that agents or factors that elevate cAMP in breast cancer cells should reduce the effectiveness of tamoxifen-like antiestrogens used in hormonal therapy of breast cancer and may lead to antiestrogen resistance. In addition, we find that antiestrogens themselves can increase cAMP levels, rendering the antiestrogens less potent antagonists of estrogen action and more potent stimulators of estrogen-induced effects, resulting in compromising of the tumor growth suppressing activities of antiestrogens. Our observations in this past year of the grant which indicate that cells resistant to the growth suppressive affects of antiestrogen (including our MCF/TOT cells or cells overexpressing ras or FGF-1 or FGF-4) contain substantially elevated levels of intracellular cAMP, are consistent

with the hypothesis that elevated cAMP levels may compromise the growth suppressive activities of antiestrogens, rendering the cells insensitive to these normally growth suppressive compounds. In contrast to mechanisms for tamoxifen resistance that involve mutations in the estrogen receptor or other critical growth regulatory genes, which would not be reversible, our proposed mechanism involving a compromising of tamoxifen effectiveness as an antiestrogen in the presence of elevated levels of intracellular cAMP, would be a progressive, adaptational response, which would be reversible upon cessation of tamoxifen therapy. Indeed, our findings in Herman and Katzenellenbogen, publication #8, and also clinical experience support a mechanism of this type in that patients who become resistant to tamoxifen often return to a state of tamoxifen responsiveness after a period of alternate therapy (during which time cAMP levels in tumor cells may drop such that newly administered tamoxifen would again be effective as a growth suppressive agent). In addition, our data could account for the observation that hormonal resistance in model mammary tumor systems develops much more slowly to ICI 164,384 than to tamoxifen in that the agonistic character of ICI 164,384 is not augmented by cAMP. Therefore, ICI 164,384-like antiestrogens may prove to be more long-term effective antiestrogens compared with tamoxifen.

In the next year of this grant, we will focus primarily on Tasks 3 and 4. We will identify and characterize the membrane binding site through which estrogen and antiestrogen stimulate adenylate cyclase in breast cancer cells, and we will determine if this a new binding protein or an estrogen receptor-like protein. We will also work toward determining the mechanism by which increased cAMP alters the biocharacter (agonist/antagonist activity) of antiestrogens. We will determine the effect of tamoxifen, ICI 164,384, and estrogen alone and in the presence of elevated levels of cAMP, on phosphorylation of the estrogen receptor, using tryptic phosphopeptide analysis and site-directed mutagenesis to identify sites of phosphorylation that may be associated with the alteration in tamoxifen agonist character. Through these mutational analyses we will determine which phosphorylation sites on the receptor are associated with changes in tamoxifen agonist character in the presence of cAMP and we will determine whether there are differences in receptor phosphorylation in parental antiestrogen-responsive versus in antiestrogen-resistant MCF-7 breast cancer sublines. These investigations should provide insight into the nature of antiestrogen resistance and the role of cAMP modulation of estrogen and antiestrogen action in hormonal resistance. We hope through our findings to provide an understanding of tamoxifen resistance at the molecular level, and thus to point towards new directions for more effective implementation of antiestrogen treatments in breast cancer patients that may prove to be more long-term and effective compared to tamoxifen.

Publications Resulting From This Research : (* indicates copy present in Appendix)

*1. Nicholson, R. I., Gee, J. M. W., Francis, A. B., Manning, D. L., Wakeling, A. E., and Katzenellenbogen, B. S. Observations arising from the use of pure antioestrogens on oestrogen-responsive (MCF-7) and oestrogen growth-independent (K3) human breast cancer cells. Endocrine Related Cancer 2:115-121, 1995.

*2. Katzenellenbogen, B. S., Montano, M., Le Goff, P., Schodin, D. J., Kraus, W. L., and Bhardwaj, B., and Fujimoto, N. Antiestrogens: Mechanisms and actions in target cells. J. Steroid Biochem. Molec. Biol. 53:387-393, 1995.

*3. Ince, B. A., Schodin, D. J., Shapiro, D. J., and Katzenellenbogen, B. S. Repression of endogenous estrogen receptor activity in MCF-7 human breast cancer cells by dominant negative estrogen receptors. Endocrinology, 136: 3194-3199, 1995.

*4. Nicholson, R. I., Gee, J. M. W., Manning, D. L., Wakeling, A. E., Montano, M. M., andBSK: slses to pure antioestrogens (ICI 164384, ICI 182780) in oestrogen sensitive and resistant experimental and clinical breast cancer. Annals N. Y. Acad. Sci. 761:148-163, 1995.

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*11. Katzenellenbogen, B. S. Estrogen receptor bioactivities and interactions with signal transduction pathways in breast cancer cells. Proceedings, Amer. Assoc. for Cancer Research 37:630, 1996

12. Katzenellenbogen, B. S. Estrogen receptor mechanisms and actions in target cells. Endocrinology 138 (Suppl.): Plenary lecture abstract L1, p.13, 1996

*13. McInerney, E. M., and Katzenellenbogen, B. S. Different regions in activation function-1 of the human estrogen receptor required for antiestrogen-dependent and estradiol-dependent transcription activation. J. Biol. Chem. 271: 24172-24178, 1996.

14. Montano, M. M., Kraus, W. L., and Katzenellenbogen, B. S. Identification of a novel transferable cis element in the promoter of an estrogen responsive gene that modulates sensitivity to hormone and antihormone. Submitted.

Appendix
Dr. Benita S.
Katzenellenbogen
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Progress Report
Year 2

Observations arising from the use of pure antioestrogens on oestrogen-responsive (MCF-7) and oestrogen growth-independent (K3) human breast cancer cells

by R I Nicholson, J M W Gee, A B Francis, D L Manning, A E Wakeling and B S Katzenellenbogen

INTRODUCTION

During the last 7 years the Breast Cancer Group within the Tenovus Cancer Research Centre has maintained an involvement in the use of pure antioestrogens in two important areas of breast cancer research. First, their development as clinical agents, where we hoped to induce total oestrogen deprivation and thereby improve the effectiveness of first-line endocrine therapy (Nicholson *et al.* 1992, Nicholson 1993, Nicholson *et al.* 1993*a*, DeFriend *et al.* 1994, Nicholson *et al.* 1994*c*). Second, as pharmacological probes to investigate the cellular and molecular actions of oestrogens and tamoxifen (Nicholson *et al.* 1988, Weatherill *et al.* 1988, Wilson *et al.* 1990). Implicit in each of these areas of research are questions associated with the impact which pure antioestrogens might have on the therapy of endocrine-resistant states and whether resistance develops as a consequence of incomplete oestrogen withdrawal, with tumour cells more efficiently utilising either a reduced oestrogenic pool or the agonistic activity of an antioestrogen, or whether the resistant cells have completely circumvented the need for oestrogen receptor (ER)-mediated growth and hence sensitivity to the antitumour properties of pure antioestrogens (Nicholson *et al.* 1994*c*).

On this basis, in the current article we seek to describe a number of the properties exhibited by pure antioestrogens in oestrogen-responsive MCF-7 human breast cancer cells (Nicholson *et al.* 1990, Nicholson *et al.* 1995) and in the oestrogen growth-independent variant K3 (Katzenellenbogen *et al.* 1987, Clarke *et al.* 1989, Cho *et al.* 1991, Reese & Katzenellenbogen 1992) of this tumour cell line. Limited data will also be presented on the growth-inhibitory properties of 4-(3-methylanilino)quinazoline (aniloquinazoline), a tyrosine kinase inhibitor which shows specificity for epidermal growth factor (EGF)-receptor signalling (Wakeling *et al.* 1994). The data presented are consistent with ER-mediated growth being important not only in MCF-7 cells, but also in their oestrogen-resistant variant, with transforming growth factor α (TGFα) possibly playing a supportive growth-regulatory role.

COMPARATIVE GROWTH EFFECTS OF OESTRADIOL AND ANTIOESTROGENS ON WILD-TYPE AND K3 MCF-7 CELLS

K3 cells were originally isolated by the exposure of MCF-7 human breast cancer cells to culture conditions low in oestrogenic substances (Katzenellenbogen *et al.* 1987). Thus, by growing MCF-7 cells in phenol red-free media and 5% dextran-coated charcoal-treated (DCC-stripped) foetal calf serum (FCS) for prolonged periods, a stable cell variant (K3) was obtained which showed a markedly increased basal rate of proliferation where added oestrogen was unable to increase this rate of

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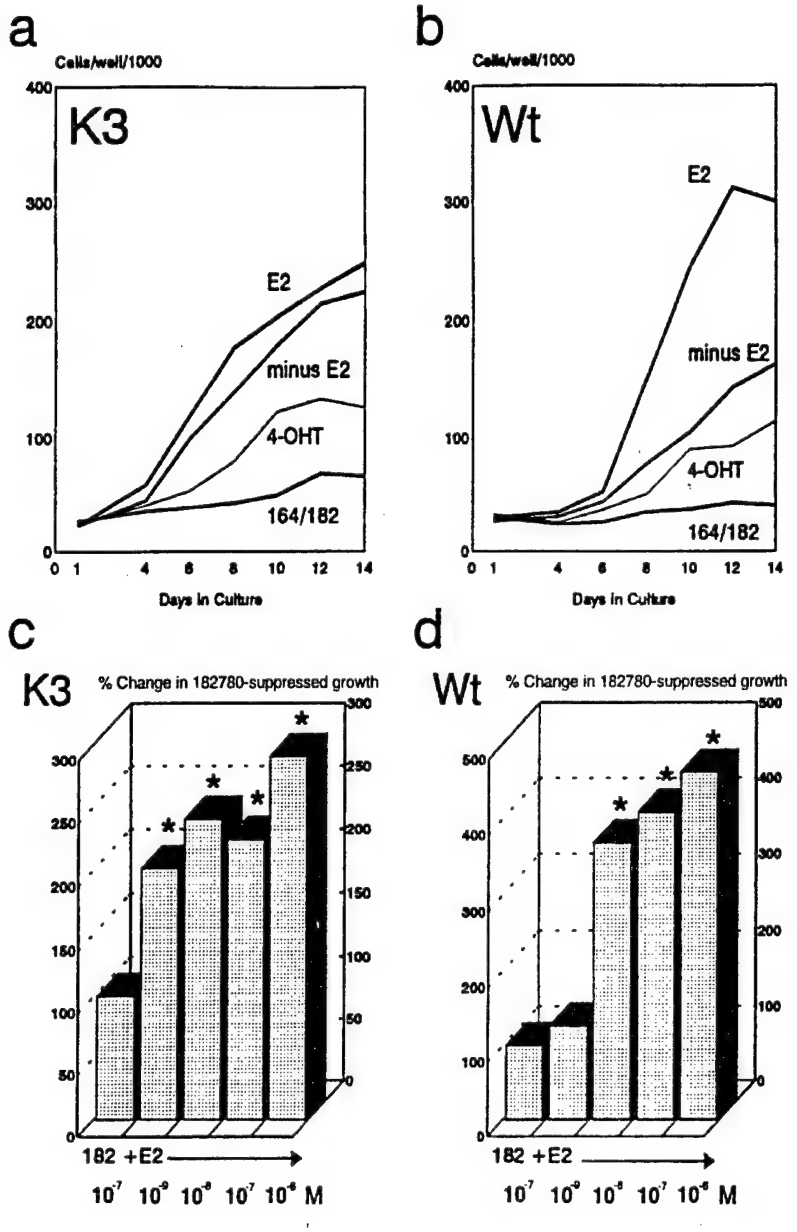

Figure 1 Characterisation of the growth of K3 and Wt MCF-7 cells in monolayer culture. (a and b) The cells were grown in multiwell dishes in white RPMI tissue culture medium with 5% DCC-stripped FCS (medium A); without additives (minus E2), and medium A containing 10^{-9}M oestradiol (E2), 10^{-7}M 4-hydroxytamoxifen (4-OHT), and 10^{-7}M ICI 182780 (164/182) for up to 14 days. (c and d) The cells were grown in medium A containing 10^{-7}M ICI 182780 for 8 days prior to the addition of various doses of oestradiol (182+E2). These cultures were harvested on day 14 after the addition of oestradiol. Cell numbers were assessed by the use of a Coulter counter and are the mean of 3 replicate cultures counted in triplicate. *P v 182<0.05: statistical analysis performed using a Mann-Whitney U test.

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THE PARADO
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proliferation further. These results are essentially duplicated in Figure 1 and contrast with the stimulatory effect of added oestradiol (10^{-9}M) on the growth of our Wt-MCF-7 cells in media lacking endogenous oestrogens.

Despite their apparent oestrogen growth-independence, early studies established that the growth of K3 cells could be inhibited by 10^{-7}M 4-hydroxytamoxifen (Katzenellenbogen *et al.* 1987, Clarke *et al.* 1989). This effect is also illustrated in Figure 1a. In the present study we have used the pure antioestrogen ICI 182780 (10^{-7}M) (Wakeling *et al.* 1991) to establish whether complete oestrogen deprivation can achieve a greater antitumour effect than can the use of antioestrogens, like tamoxifen, with partial oestrogen-like activity (Nicholson *et al.* 1995). Figure 1a shows the growth-inhibitory activity of ICI 182780 exceeding that of 4-hydroxytamoxifen, allowing at maximum 2 doublings of the initial cell number. Over several experiments we have estimated the tumour cell doubling time for ICI 182780-treated K3 and wild-type (Wt) cells to be in excess of 150h. This contrasts with 32-35h for oestrogen-treated and oestrogen-withdrawn K3 cells (Katzenellenbogen *et al.* 1987, Clarke *et al.* 1989) and >80h for 4-hydroxytamoxifen-treated cells (Katzenellenbogen *et al.* 1987).

Importantly, the improved level of growth inhibition shown by pure antioestrogens in several breast tumour cell lines appears specific for ER signalling, in that their actions are restricted to ER-positive cancer cells and they are achieved at molar concentrations (10^{-10} to 10^{-9}) equivalent to the dissociation constant for their binding to ER. Moreover, the actions of antioestrogens may be reversed by oestradiol (see refs in Nicholson *et al.* 1994*a*). This property is demonstrated for pure antioestrogens both in K3 and in Wt cells in Figure 1c and d. Indeed, ICI 182780 growth-suppressed K3 cells show an increased sensitivity to oestradiol in comparison with wild-type cells, with the effects of 10^{-7}M ICI 182780 reversed by 10^{-9}M oestradiol.

THE PARADOX AND A POTENTIAL SOLUTION

These data represent a paradox both for K3 and for Wt cells, each of which are capable of growth in the apparent absence of oestradiol (K3>Wt), yet are growth inhibited by a pure antioestrogen whose perceived mechanism of action is to antagonise the cellular actions of oestrogens at the ER. Indeed, their inhibitory actions may be reversed (K3>Wt) by oestradiol. A potential solution to this paradox arises from the observation that the cellular actions of the ER, in either an occupied (Wakeling *et al.* 1991, refs in Nicholson *et al.* 1994*a*) or unoccupied (Ignar-Trowbridge *et al.* 1992) form, may be potentiated by the presence of growth factors. ER-induced growth responses, therefore, may require only limited amounts of steroid, with differences between K3 and Wt cells reflecting altered regulation of growth factor production or cellular sensitivity to their actions.

AN INVOLVEMENT OF TGFα?

As may be seen in Figure 2, when grown in an oestrogen-depleted environment K3 cells show a higher basal expression of the mitogenic growth factor TGFα than do Wt cells. Furthermore, in K3 cells the intracellular level of this protein is only poorly induced by oestradiol compared with a twofold increase seen in Wt cells. This parallels the lack of activity of the steroid on K3 growth. In each instance, ICI 182780 reduced the basal expression of TGFα. Importantly, the reduction in TGFα levels in pure antioestrogen-treated cells accompanies a substantial fall in their ER content (Fig. 2c and d; Reese & Katzenellenbogen 1992). This action would minimise the opportunity for cross talk between ER signalling and TGFα signalling pathways. Interestingly, K3 cells also show an elevated basal expression of pS2 (Cho *et al.* 1991), a protein whose gene promoter contains response elements both for oestradiol and for TGFα (Nunez *et al.* 1989). Once again, the expression of this protein is efficiently reduced by the presence of the pure antioestrogen (Nicholson *et al.* 1995).

Finally, we have examined the effects of 4-(3-methylanilino)quinazoline (ZM163613), a tyrosine kinase inhibitor reported to show specificity for EGF-receptor signalling (Wakeling *et al.* 1994, Ward *et al.* 1994), on K3 and Wt cells in order to determine whether TGFα is directly involved in growth signalling and oestrogen-regulated gene expression. The data shown in Figure 3a and b show that the Wt cells

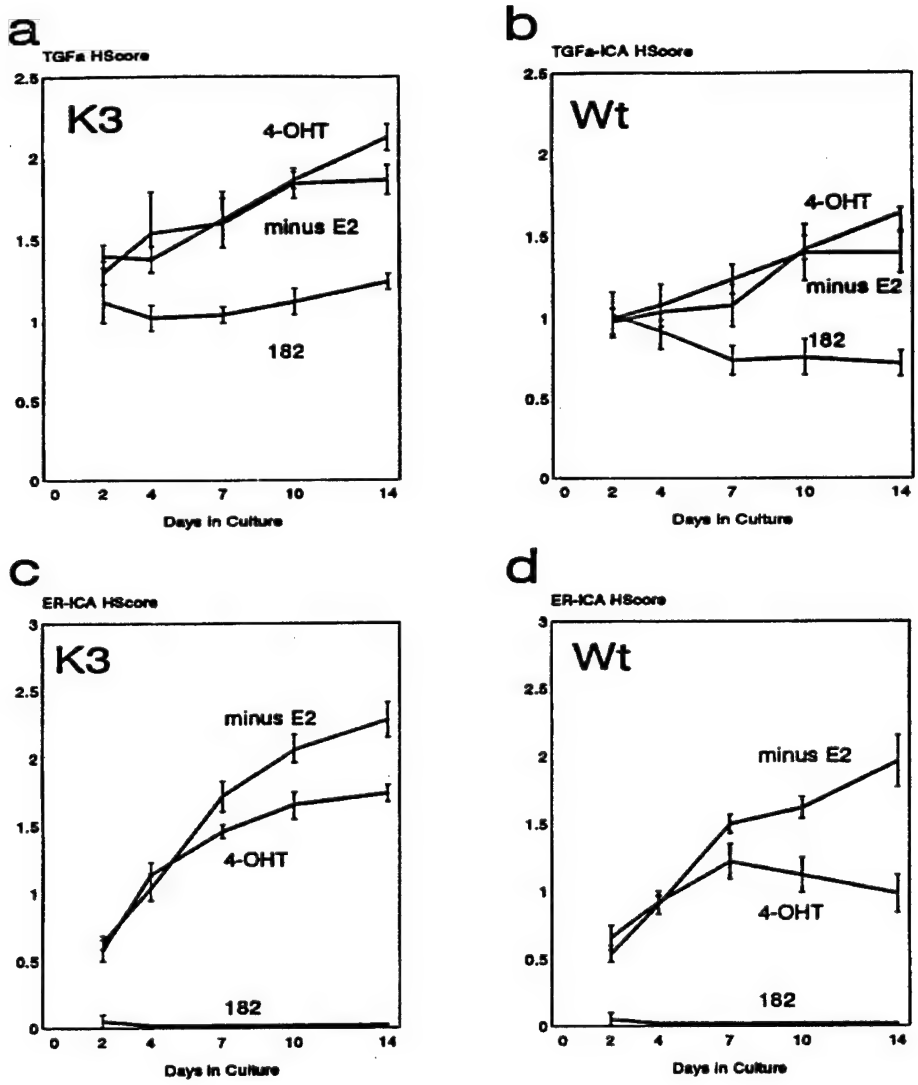

Figure 2 Immunohistochemical characterisation of K3 and Wt MCF-7 cells. The cells were cultured on 3-aminopropyltriethoxysilane-coated glass coverslips in medium A containing no additions (minus E2), 10^{-9}M oestradiol (E2), 10^{-7}M 4-hydroxytamoxifen (4-OHT), and 10^{-7}M ICI 182780 (182) for up to 14 days. TGFα (a and b) and ER (c and d) assays were performed according to the methods of Nicholson *et al.* (1991, 1993*b*) and Walker *et al.* (1988), respectively. The results are shown as mean values±S.D. of 5 replicates from a minimum of 2 coverslips. H scores were calculated according to the method of Gee *et al.* (1994).

are strongly growth inhibited by the drug at a concentration of 10μM. At this concentration, the cells show reduced basal progesterone-receptor and pS2 levels whilst maintaining ER and TGFα cellular concentrations (Nicholson *et al.* 1995). However, an identical dose of ZM163613 is less growth inhibitory to K3 cells (Fig. 3a) and does not alter oestrogen-regulated gene expression, although some growth

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CONCLUSIONS

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inhibition of K3 cells (Fig. 3a) and decrease in pS2 (RI Nicholson, unpublished observations) may be achieved by 50μM ZM163613.

CONCLUSIONS

Several conclusions may be arrived at on the basis of the results presented.

(1) The importance of ER-mediated signalling is retained in the basal growth responses of oestrogen growth-independent K3 cells and is in parallel with observations made on tamoxifen-resistant tumours which are sensitised to the agonistic activity of the drug (Osborne *et al.* 1994).

(2) TGFα signalling may impinge on ER-mediated growth and circumvent the need for high oestrogen levels. This response may be exaggerated in K3 cells, potentially decreasing the cellular sensitivity to ZM163613.

(3) Pure antioestrogens antagonise ER-mediated effects, in Wt and K3 cells, possibly by decreasing ER and TGFα levels and thereby reducing cross talk between these growth-signalling pathways.

Finally, it is interesting that we have also observed that a failure of ER-positive advanced breast cancer to respond to antihormones correlates with elevated TGFα levels (Nicholson *et al.* 1994*b*) and elevated cell-proliferation rates, evidenced by an increased Ki67 immunostaining (Nicholson *et al.* 1991, Nicholson *et al.* 1993*b*); factors which in K3 cells are associated with acquired oestrogen growth-independence. If these factors are causative in the loss of oestrogen growth-responsiveness, then primary and acquired endocrine resistance may occur on a similar developmental pathway and be equally vulnerable to pure antioestrogens. Trials to examine these possibilities are awaited.

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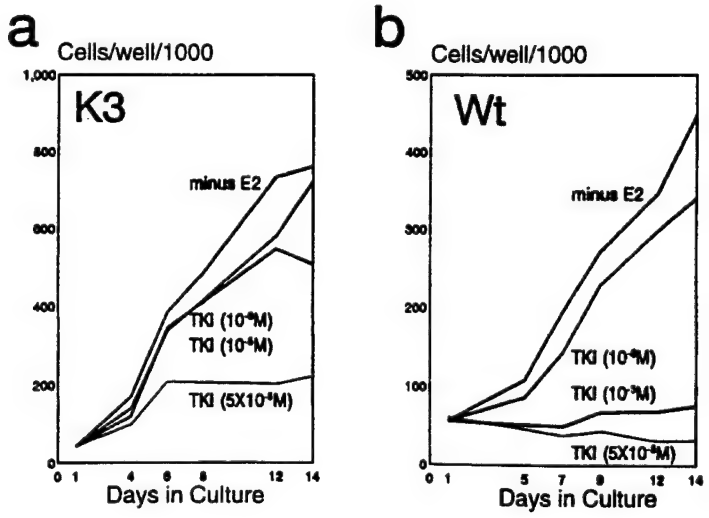

Figure 3 Effect of 4-(3-methylanilino)quinazoline on the growth of K3 and Wt cells. The cells were grown as described in Figure 1, in medium A alone (minus E2) or containing the stated dose of the tyrosine kinase inhibitor (TKI). The results presented are the mean of 3 replicate cultures counted in triplicate.

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Antiestrogens: Mechanisms and Actions in Target Cells

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Antiestrogens, acting via the estrogen receptor (ER) evoke conformational changes in the ER and inhibit the effects of estrogens as well as exerting anti-growth factor activities. Although the binding of estrogens and antiestrogens is mutually competitive, studies with ER mutants indicate that some of the contact sites of estrogens and antiestrogens are likely different. Some mutations in the hormone-binding domain of the ER and deletions of C-terminal regions result in ligand discrimination mutants, i.e. receptors that are differentially altered in their ability to bind and/or mediate the actions of estrogens vs antiestrogens. Studies in a variety of cell lines and with different promoters indicate marked cell context- and promoter-dependence in the actions of antiestrogens and variant ERs. In several cell systems, estrogens and protein kinase activators such as cAMP synergize to enhance the transcriptional activity of the ER in a promoter-specific manner. In addition, cAMP changes the agonist/antagonist balance of tamoxifen-like antiestrogens, increasing their agonistic activity and reducing their efficacy in reversing estrogen actions. Estrogens, and antiestrogens to a lesser extent, as well as protein kinase activators and growth factors increase phosphorylation of the ER and/or proteins involved in the ER-specific response pathway. These changes in phosphorylation alter the biological effectiveness of the ER. Multiple interactions among different cellular signal transduction systems are involved in the regulation of cell proliferation and gene expression by estrogens and antiestrogens.

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INTRODUCTION: ESTROGEN TARGET TISSUES AND ANTIESTROGEN EFFECTIVENESS

Estrogens influence the growth, differentiation and functioning of many target tissues. These include tissues of the reproductive system such as the mammary gland and uterus, cells in the hypothalamus and pituitary, as well as bone where estrogens play important roles in bone maintenance; and the liver and cardiovascular systems where estrogens influence liver metabolism, the production of plasma lipoproteins, and exert cardioprotective effects [1–3]. Estrogens, in addition to stimulating mammary gland growth and duct development, also increase proliferation and metastatic activity of breast cancer cells [4] and stimulate the proliferation of uterine cells [1]. Antiestrogens, which antagonize the actions of estrogens, therefore have much potential as important therapeutic agents. Our studies have examined the effects of antiestrogens on a variety of target cells including liver [5] and hypothalamus and pituitary [6], but have primarily focused on their effects on breast cancer and uterine cells [7].

The actions of estrogens on breast cancer and uterine cells are antagonized by antiestrogens, which bind to the estrogen receptor (ER) in a manner that is competitive with estrogen but they fail to effectively activate gene transcription [7–9]. Two of the major challenges in studies on antiestrogens are to understand what accounts for their antagonistic effectiveness as well as the partial agonistic effects of some antiestrogens; and to understand how one can achieve tissue selective agonistic/antagonistic effects of these compounds. One of our approaches to addressing these issues has been to try to understand in detail how the ER discriminates between

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estrogen and antiestrogen ligands and between different categories of antiestrogens. This has involved the generation and analysis of variant human ERs with mutations throughout the ER hormone-binding domain and study of the activity of these receptors on different estrogen-responsive genes in several cell backgrounds when liganded with antiestrogen or estrogen. These studies and those of others have provided consistent evidence for the promoter-specific and cell-specific actions of the estrogen-occupied and antiestrogen-occupied ER. In addition, in the studies described below, we have observed that protein kinase activators enhance the transcriptional activity of the ER and alter the agonist/antagonist balance of some antiestrogens, suggesting that changes in cellular phosphorylation state should be important in determining the effectiveness of antiestrogens as estrogen antagonists.

ANALYSIS OF THE ER HORMONE BINDING DOMAIN AND LIGAND DISCRIMINATION

We have examined the interactions of estrogen and antiestrogens with the ER and the modulation of ER activity by phosphorylation and interaction with other proteins which result in changes in ER-mediated responses. Studies by us [10–17] have provided strong documentation that the response of genes to estrogen and antiestrogen depend on four important factors: (1) the nature of the ER, i.e. whether it is wild-type or variant; (2) the promoter; (3) the cell context; and (4) the ligand. The gene response, in addition, can be modulated by cAMP, growth factors, and agents that affect protein kinases and cell phosphorylation [15, 18–21]. These may account for differences in the relative agonism/antagonism of antiestrogens like tamoxifen on different genes and in different target cells such as those in breast cancer cells, versus uterus, versus bone.

Antiestrogens are believed to exert their effects in large measure by blocking the actions of estrogens by competing for binding to the ER and altering ER conformation such that the receptor fails to effectively activate gene transcription. In addition, antiestrogens exert anti-growth factor activities, via a mechanism that requires ER but is still not fully understood [22].

Models of antiestrogen action at the molecular level are beginning to emerge, and recent biological studies as well indicate that antiestrogens fall into two distinct categories: antiestrogens, such as tamoxifen, that are mixed or partial agonists/antagonists (type I), and compounds, such as ICI 164,384, that are complete/pure antagonists (type II). The type I antihormone-ER complexes appear to bind as dimers to estrogen response elements (EREs); there, they block hormone-dependent transcription activation mediated by region E of the

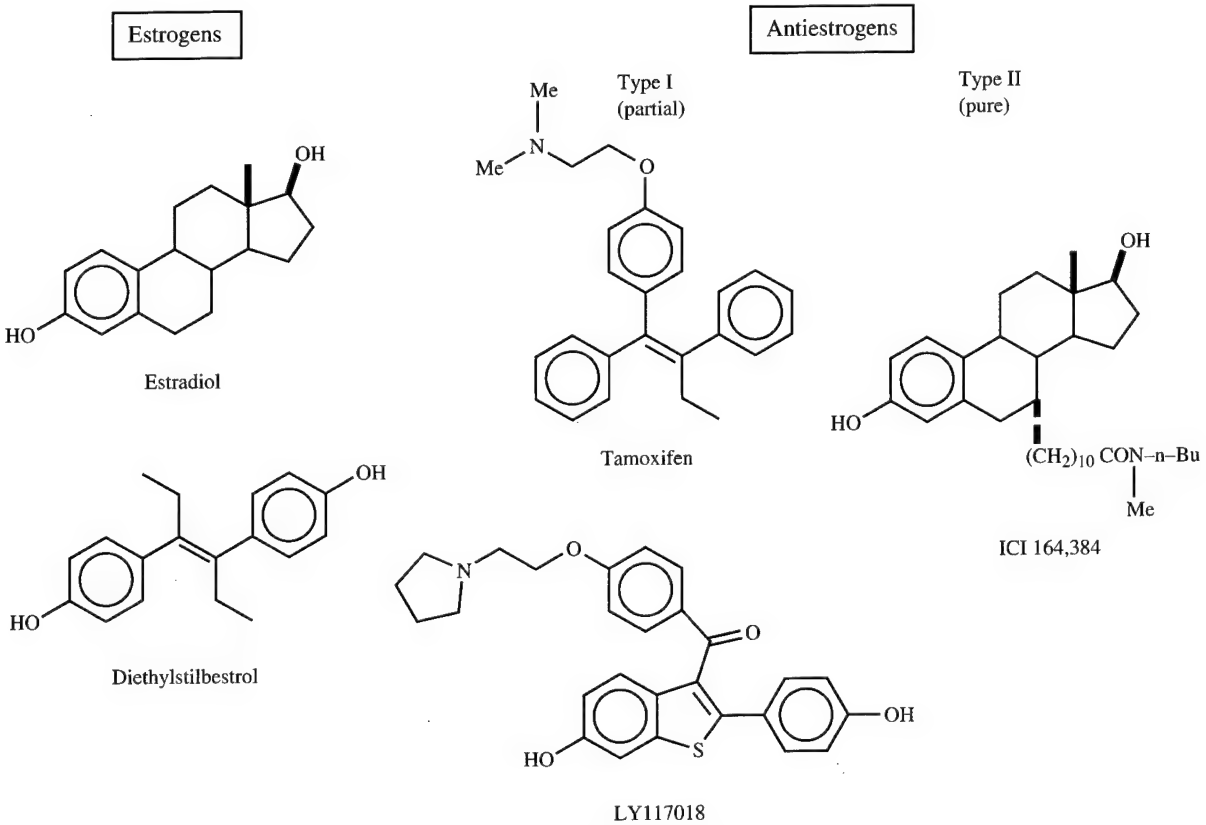

Fig. 1. Structures of several estrogenic and antiestrogenic ligands for the estrogen receptor used in our studies. The antiestrogens include the nonsteroidal compounds tamoxifen and LY117018 that often show partial agonist/antagonist activity (type I antiestrogens) and the steroidal, more pure antiestrogen ICI164,384 (type II antiestrogen).

receptor, but are believed to have little or no effect on the hormone-independent transcription activation function located in region A/B of the receptor [16]. Thus, they are generally partial or mixed agonist/antagonists, and their action must involve some subtle difference in ligand–receptor interaction, very likely associated with the basic or polar side chain that characterizes the antagonist members of this class. In the case of the more complete antagonists, such as ICI 164,384, obstruction of ER binding to DNA and reduction of the ER content of target cells appear to contribute to [23, 24], but may not fully explain, the pure antagonist character of this antiestrogen [25]. The structures of these antiestrogens, which can be both steroidal or non-steroidal in nature, are shown in Fig. 1, along with the structures of the naturally occurring estrogen estradiol, and the non-steroidal synthetic estrogen diethylstilbestrol. Of note, is the fact that antiestrogens typically have a bulky side chain which is basic or polar. This side chain is important for antiestrogenic activity; removal of this side chain results in a compound which is no longer an antiestrogen and, instead, has only estrogenic activity. Therefore we believe that interaction of this side chain with the ER must play an important role in the interpretation of the ligand as an antiestrogen.

In order to examine issues of ligand discrimination by the ER, we have used site-directed and random chemical mutagenesis to generate ERs with selected changes in the hormone binding domain. We have been particularly interested in identifying residues in the hormone binding domain important for the ligand binding and transactivation functions of the receptor, and in elucidating the mechanism by which the ER discriminates between agonistic and antagonistic ligands. Although both estrogens and antiestrogens bind within the HBD, the association must differ because estrogen binding activates a transcriptional enhancement function, whereas antiestrogens fully or partially fail in this role. Our studies have indicated that selective changes near amino acid 380, and amino acids 520–530, and changes at the C-terminus of the ER result in ER ligand discrimination mutants [10, 13, 26]. These data provide evidence that some contact sites of the receptor with estrogen and antiestrogen differ; and that the conformation of the receptor with estrogen and antiestrogen must also be different as a consequence [10, 27 and refs therein]. Our structure–function analysis of the hormone binding domain of the human ER has utilized region-specific mutagenesis of the ER cDNA and phenotypic screening in yeast, followed by the analysis of interesting receptor mutants in mammalian cells [14, 28]. Our observations, as well as very important studies by Malcolm Parker and colleagues [29, 30] have shown a separation of the transactivation and hormone-binding functions of the ER.

Since the basic or polar side chain is essential for antiestrogenic activity, and our previous studies identified cysteine 530 as the amino acid covalently labeled by affinity labeling ligands [31], we introduced by site directed mutagenesis of the ER cDNA changes of specific charged residues close to C530 [10]. Interestingly, two mutants in which lysines at position 529 and 531 where changed to glutamines, so that the local charge was changed, resulted in receptors with an approx. 30-fold increased potency of antiestrogen in suppressing estradiol-stimulated reporter gene activity. Interestingly, these mutants receptors showed a reduced binding affinity for estrogens, but retained unaltered binding affinity for antiestrogen. These findings suggest that we are able to differentially alter estrogen and antiestrogen effectiveness by rather modest changes in the ER, and that the region near C530 is a critical one for sensing the fit of the side chain of the estrogen antagonist. Studies from the Parker Laboratory [27] have shown that nearby residues (i.e. G525 and M521 and/or S522 in the mouse ER) are also importantly involved in conferring differential sensitivity to these two categories of ligands.

We have also shown that if C530 is mutated, the covalent ligand tamoxifen aziridine binds to C381 instead, another cysteine in the hormone binding domain [32]. One interpretation of this result is that the 530 and 380 regions of the hormone-binding domain are close to one another in the three-dimensional ligand binding pocket of the ER, such that the ligand can label either site by alternative positioning of the reactive side chain [32]. We therefore investigated charged amino acids in the N-terminal portion of the hormone binding domain and showed the region around amino acid 380 to be important in transcriptional activity of the receptor [13]. As opposed to what was observed with charge changes in the region near C530, we observed that change of the charged residue E380 to E380Q resulted in a receptor more sensitive to estrogen, but less sensitive than wild-type ER to antiestrogen for suppression of transcriptional activity. Although estrogen and antiestrogen showed no alteration of their binding affinity for the wild-type or E380Q mutant, the E380 mutant showed greater transcriptional activity and enhanced binding to estrogen response element DNA, resulting in its increased sensitivity to estrogen. Our findings suggest that this region is important in influencing DNA binding and protein–protein interaction of the receptor that modulates transcriptional activity and provide additional evidence, suggesting that the conformation of the receptor with estrogen and antiestrogen results in differential transactivation activity. Our recent data [26] has also shown that tamoxifen-like antiestrogens are more pure antiestrogens with the ER missing the C-terminal F domain, approx. the last 40 amino acids of the receptor. The basis for the difference in the estrogenic activity of tamoxifen-like estrogens with wild-type ER versus ER missing this F domain is under investigation and should provide important information regarding the differential agonistic/antagonistic effects of this category of antiestrogens.

ALTERATION IN THE AGONIST/ANTAGONIST BALANCE OF ANTIESTROGENS BY ACTIVATION OF PROTEIN KINASE A SIGNALING PATHWAYS: ANTIESTROGEN SELECTIVITY AND PROMOTER DEPENDENCE

There is increasing evidence for ER interaction with other cell signaling pathways. We became interested in this cross-talk between cell signaling pathways in our studies of estrogen regulation of the progesterone-receptor and estrogen responsive promoter-reporter gene constructs in cells. These studies showed stimulation by growth factors (IGF-1, EGF) as well as stimulation by cAMP and estrogen. The observation that the stimulation by these agents could be suppressed by antiestrogens or protein kinase inhibitors implied the involvement of the ER and phosphorylation pathways in these responses [18–21, 33]. We therefore have undertaken studies to examine directly whether activators of protein kinases can modulate transcriptional activity of the ER.

We find that activators of protein kinase A and protein kinase C markedly synergize with estradiol in ER-mediated transcriptional activation and that this transcriptional synergism shows cell- and promoter-specificity [15, 21, 34]. The synergistic stimulation of ER-mediated transcription by estradiol and protein kinase activators did not appear to result from changes in ER content or in the binding affinity of ER for ligand or the ERE DNA, but, rather, may be a consequence of a stabilization or facilitation of interaction of target components of the transcriptional machinery, possibly either through changes in phosphorylation of ER or other proteins important in ER-mediated transcriptional activation [34].

Figure 2 shows a model indicating how we think the protein kinase–ER transcriptional synergism may occur. Agents influencing protein kinase pathways may enhance intracellular protein phosphorylation resulting in either phosphorylation of the ER itself or the phosphorylation of nuclear factors with which the receptor interacts in mediating transcription. Likewise, there is evidence that the steroid hormone itself can alter

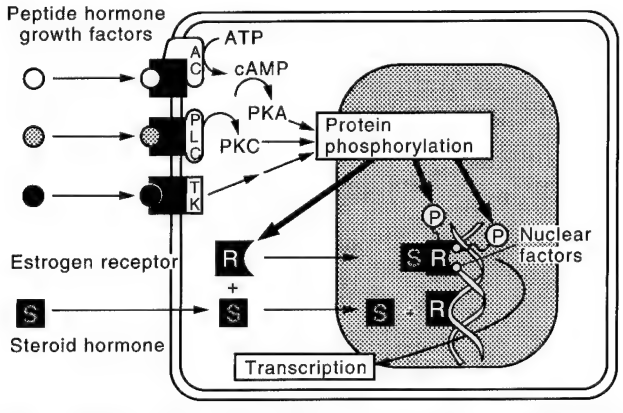

Fig. 2. Model depicting protein kinase–ER transcriptional synergism. See text for description.

Table 1. Levels of ligand-stimulated and protein kinase activator-stimulated phosphorylation of the human ER

Treatments	Phosphorylation level mean ± SE	*n*
Control	1	
10^{-9} M estradiol (E_2)	2.8 ± 0.3	3
10^{-8} M estradiol (E_2)	4.3 ± 0.7	6
10^{-8} M transhydroxytamoxifen (TOT)	2.9 ± 0.1	2
10^{-7} M ICI 164,384	3.6 ± 0.6	3
1 μg/ml cholera toxin (CT) + 10^{-4} M isobutylmethylxanthine (IBMX)	1.9 ± 0.3	3
10^{-7} M TPA	2.6 ± 0.3	3

Human ER was expressed in COS-1 cells and transfected cells were incubated for 4 h with [^{32}P]orthophosphate in the presence of the indicated treatment. ER was immunoprecipitated with anti-receptor antibodies, resolved by SDS–PAGE and transferred to nitrocellulose. ER protein levels were determined by immunoblot and ER phosphorylation by autoradiography as described [35]. The levels of phosphorylation of the different samples were standardized according to ER protein levels and standard errors (SE) were calculated. 1 represents the basal level of phosphorylation (vehicle alone) in each experiment. *n* represents the number of experiments. (From Le Goff *et al.* ref. [35]).

receptor conformation increasing its susceptibility to serve as a substrate for protein kinases [19, 35–38 and Table 1]. Therefore, agents which increase the phosphorylation may, either through phosphorylation of the ER itself, or through phosphorylation of nuclear factors required for ER transcription, result in synergistic activation of ER-mediated transcription.

As shown in Fig. 3, we have compared the effects of cAMP on the transcriptional activity of the estradiol-liganded and antiestrogen-liganded ER complexes. We find that increasing the intracellular concentration of cAMP, or of protein kinase. A catalytic subunit of transfection [15], activates and/or enhances the transcriptional activity of type I but not type II antiestrogen-occupied ER complexes and reduces the estrogen antagonist activity of the type I transhydroxytamoxifen (TOT) antiestrogen. In Fig. 3(A and B), we have determined, in MCF-7 human breast cancer cells, the effect of cAMP on the activity of TOT, ICI 164,384 and E_2 on a simple TATA promoter with one consensus ERE upstream of the CAT gene and on the more complex pS2 gene promoter and 5′-flanking region (−3000 to +10) containing an imperfect ERE. The endogenous pS2 gene is regulated by E_2 in MCF-7 breast cancer cells. Estradiol increased the transcription of both of these gene constructs, and treatment with IBMX/CT and E_2 evoked a synergistic increase in transcription, with activity being ca 2.5 times that of E_2 alone. Both antiestrogens (TOT and ICI) failed to stimulate transactivation of these reporter gene constructs, but in the presence of IBMX/CT, TOT gave significant stimulation of transcription (85 or 60% that of E_2 alone). ICI failed to stimulate transactivation even in the presence of IBMX/CT, and ICI fully blocked E_2 stimulation in the presence or absence of cAMP. By contrast, treatment with IBMX/CT reduced the ability

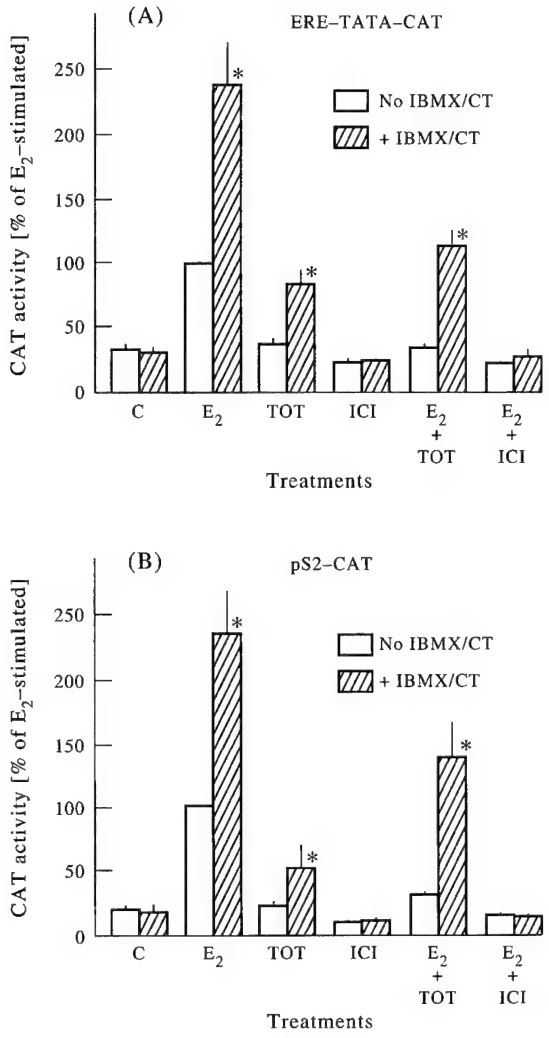

Fig. 3. Effect of IBMX/CT on the ability of E_2 and antiestrogens to stimulate transactivation of ERE-TATA-CAT (panel A) and pS2-CAT (panel B), and on the ability of antiestrogens to suppress E_2-stimulated transactivation. MCF-7 cells were transfected with the indicated reporter plasmid and an internal control plasmid that expresses β-galactosidase and were treated with the agents indicated for 24 h. Each bar represents the mean ± SEM ($n = 3$ experiments). * Indicates significant difference from the no IBMX/CT cells ($P < 0.05$). C, control ethanol vehicle; E_2, 10^{-9} M; TOT (hydroxytamoxifen), 10^{-6} M; ICI (ICI 164,384), 10^{-6} M; IBMX (3-isobutyl-1-methyl-xanthine), 10^{-4} M; and CT (cholera toxin), 1 μg/ml. (From Fujimoto and Katzenellenbogen, ref. [15]).

of TOT to inhibit E_2 transactivation. While TOT returned E_2 stimulation down to that of the control in the absence of IBMX/CT (compare open bars E_2 vs E_2 + TOT), TOT only partially suppressed the E_2 stimulation in the presence of IBMX/CT (compare stippled bars E_2 vs E_2 + TOT).

Although alteration in the agonist and antagonist activity of TOT was observed with promoter-reporter-constructs containing a simple TATA promoter and a more complex, pS2 promoter, elevation of cAMP did not enhance the transcription by either TOT or estradiol of the reporter plasmid ERE-thymidine kinase-CAT [15]. Thus, this phenomenon is promoter-specific. Of note, cAMP and protein kinase A catalytic subunit transfection failed to evoke transcription by the more pure antiestrogen ICI 164,384 with any of the promoter-reporter constructs tested. These findings, which document that stimulation of the protein kinase A signaling pathway activates the agonist activity of tamoxifen-like antiestrogens, may in part explain the development of tamoxifen resistance by some ER-containing breast cancers. They also suggest that the use of antiestrogens like ICI 164,384, that fail to activate ER transcription in the presence of cAMP, may prove more effective for long-term antiestrogen therapy in breast cancer.

PHOSPHORYLATION OF THE ESTROGEN RECEPTOR

Since our data suggested that estrogens, and agents that activate protein kinases, might influence ER transcription by altering the state of phosphorylation of the ER and/or other factors required for ER regulation of transcription, we undertook studies to examine directly the effects of these agents on ER phosphorylation. In addition, we compared the effects of the type I and type II antiestrogens on phosphorylation of the ER (Table 1). Estradiol, each of the two antiestrogens, as well as protein kinase A and C activators enhanced overall ER phosphorylation, and in all cases, this phorphorylation occurred exclusively on serine residues [35]. Tryptic phosphopeptide patterns of wild-type and domain A/B-deleted receptors and site-directed mutagenesis of several serines involved in known protein kinase consensus sequences allowed us to identify serine 104 and/or serine 106 and serine 118, all three being part of a serine–proline motif, as major ER phosphorylation sites. Mutation of these serines to alanines so as to eliminate the possibility of their phosphorylation, resulted in an approx. 40% reduction in transactivation activity in response to estradiol while mutation of only one of these serines showed an approx. 15% decrease in activation [35]. Of note, estradiol and antiestrogen-occupied ERs showed virtually identical two-dimensional phosphopeptide patterns suggesting similar sites of phosphorylation. In contrast, the cAMP-stimulated phosphorylation likely occurs on different phosphorylation sites as indicated by some of our mutational studies [35] and this aspect remains under investigation in our laboratory.

cAMP-DEPENDENT SIGNALING PATHWAY INVOLVEMENT IN ACTIVATION OF THE TRANSCRIPTIONAL ACTIVITY OF ERs OCCUPIED BY TAMOXIFEN-LIKE BUT NOT ICI 164,384-LIKE ANTIESTROGENS

Our data provide strong evidence for the involvement of cAMP-dependent signaling pathways in the agonist actions of tamoxifen-like estrogen antagonists. The promoter-specificity of the transcriptional enhancement

phenomenon suggests that factors in addition to ER are probably being modulated by protein kinase A pathway stimulation. The findings imply that changes in the cAMP content of cells, which can result in activation of the agonist activity of tamoxifen-like antiestrogens, might account, at least in part, for the resistance to antiestrogen therapy that is observed in some breast cancer patients. Of interest, MCF-7 cells transplanted into nude mice fail to grow with tamoxifen treatment initially, but some hormone-resistant cells grow out into tumors after several months of tamoxifen exposure [8, 39, 40]. Studies have shown that this resistance to tamoxifen is, more correctly, a reflection of tamoxifen stimulation of proliferation, representing a change in the interpretation of the tamoxifen–ER complex and its agonist/antagonist balance. It is of interest that we found the pS2 gene, which is under estrogen and antiestrogen regulation in breast cancer [41], to be activated by tamoxifen in the presence of elevated cAMP. By contrast, however, antiestrogens such as ICI, shown in many systems to be more complete estrogen antagonists, are not changed in their agonist/antagonist balance by increasing intracellular concentrations of cAMP. Therefore, ICI-like compounds may prove to be more efficacious and less likely to result in antiestrogen-stimulated growth.

The transcriptional enhancement we have observed between protein kinase A activators and ER occupied by tamoxifen-like antiestrogens and estradiol provides further evidence for cross-talk between the ER and signal transduction pathways regulated by cAMP that are important in ER-dependent responses.

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Repression of Endogenous Estrogen Receptor Activity in MCF-7 Human Breast Cancer Cells by Dominant Negative Estrogen Receptors*

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ABSTRACT

We have investigated the ability of several transcriptionally inactive estrogen receptor (ER) mutants to block endogenous ER-mediated transcription in MCF-7 human breast cancer cells. In transient transfections of MCF-7 cells, two of the mutants, a frame-shifted ER (S554fs) and a point-mutated ER (L540Q), strongly inhibit the ability of endogenous wild-type ER to activate transcription of estrogen-regulated reporter plasmids. A third mutant, ER1–530, which is missing 65 residues from its carboxy-terminus, is a weaker repressor of estradiol-stimulated transcription. When an estrogen response element (ERE)-thymidine kinase-chloramphenicol acetyltransferase reporter gene is used, S554fs, L540Q, and ER1–530 suppress the transcriptional activity of endogenous MCF-7 ER by 87%, 97%, and 62%, respectively. The magnitude of dominant negative repression is promoter specific; when an ERE-pS2-chloramphenicol acetyltransferase reporter is employed, inhibition of endogenous ER activity by equivalent amounts of S554fs, L540Q, and ER1–530 ranges from 85–97%. Dose-response studies show the S554fs mutant to be the most potent of the three ER mutants as a repressor of estrogen action in these cells. In addition, elevated levels of intracellular cAMP, achieved by the addition of 3-isobutyl-1-methylxanthine plus cholera toxin to cells, fail to compromise the effectiveness of these mutants as dominant negative ERs despite the cAMP-enhanced transcriptional activity of ER. The mutants are also powerful repressors of the agonist activity of *trans*-hydroxytamoxifen-stimulated ER transcription. The dominant negative activity of the three mutants is lost when the A/B domain of these receptors is deleted, implying an important role for this N-terminal region of the ER in the ability of these mutants to inhibit endogenous wild-type ER activity. All in all, the data suggest that S554fs in particular is a reasonable candidate for studies designed to use a dominant negative ER to inhibit the estrogen- and tamoxifen-stimulated growth of human breast cancer cells. (*Endocrinology* **136:** 3194–3199, 1995)

THE GROWTH of nearly 40% of all human breast tumors is highly dependent upon the sex steroid hormone, estrogen (1–3). As the proliferative effect of estrogens on breast cancer cells is mediated by the estrogen receptor (ER), there is much interest in exploring the means by which this protein can be functionally inactivated. We are currently investigating the possibility of eventually employing dominant negative ER mutants to block wild-type ER-mediated transcription and growth stimulation in estrogen-dependent breast cancer cells.

The ER, which belongs to the conserved superfamily of steroid and thyroid receptors, is a nuclear regulatory protein that functions as a hormone-activated transcription factor in target cells (4, 5). Receptor activation is apparently a consequence of ligand-induced conformational changes in ER structure (6). The hormone-receptor complex binds with high affinity to a well defined palindromic nucleotide sequence, the estrogen response element (ERE), which is usually located upstream of an estrogen-responsive gene (7, 8). It appears that activated receptors recruit transcription factors and establish transcriptionally productive protein-protein interactions with other components of the transcription machinery (9–11). Current attempts to functionally inactivate the ER in *in vivo* and *in vitro* experimental systems and in actual breast cancer therapy employ the antiestrogen, tamoxifen. Tamoxifen binds to the ER and is thought to induce a conformational change that renders the receptor virtually incapable of activating transcription of genes involved in cancer cell proliferation and tumorigenesis (12). Administered antiestrogens have been found, however, to retain estrogenic activity in certain tissues, including some cancerous mammary tissues (13). We wanted to explore the feasibility of employing dominant negative ER mutants to suppress ER-mediated transcription, whether 17β-estradiol (E_2) or tamoxifen stimulated, in estrogen-responsive breast cancer cells.

Dominant negative mutants of a protein, when coexpressed with the wild-type version, block the action of the parent protein (14–16). Our group previously reported the successful generation of three dominant negative ER mutants and their characterization in ER-deficient Chinese hamster ovary (CHO) cells (17). In these experiments, we investigated the effectiveness of the reported mutants as inhibitors of endogenous ER in an E_2-stimulated human breast cancer cell line. We also examined the issue of dominant negative inhibition of tamoxifen-stimulated ER

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transcription, assessed the ability of the ER mutants to repress estrogen action in the presence of elevated levels of intracellular cAMP, and examined the role of the N-terminal portion of the ER in dominant negative ER activity. These studies should prove informative in efforts to identify ER mutants that can plausibly be employed in future efforts to antagonize the estrogen- and tamoxifen-stimulated growth of human breast cancer cells.

Materials and Methods

Chemicals and materials

Cell culture media and sera were purchased from Gibco (Grand Island, NY). Radioinert E_2, 3-isobutyl-1-methylxanthine (IBMX), cholera toxin (CT), and chloramphenicol were obtained from Sigma Chemical Co. (St. Louis, MO). The antiestrogen *trans*-4-hydroxytamoxifen (TOT) was provided by ICI Pharmaceuticals (Macclesfield, UK). [^{3}H]Acetyl coenzyme A (1 mCi/ml) was obtained from DuPont-New England Nuclear (Boston, MA).

Plasmids

For transcriptional activation studies, the estrogen-responsive plasmids ERE-tk-chloramphenicol acetyltransferase (CAT) (18) and $(ERE)_2$-pS2-CAT were employed. $(ERE)_2$-pS2-CAT was constructed by W. Lee Kraus of this laboratory by cloning two copies of a consensus estrogen-responsive element into the *Bam*HI site of pS2-CAT (19). Mutant human ER complementary DNAs subcloned into the eukaryotic expression vector pCMV5 (CMV = cytomegalovirus) (20) were used to express ER mutants in transfected cells. The plasmid pCH110 (Pharmacia LKB Biotechnology, Piscataway, NJ), which contains the β-galactosidase gene, was used as an internal control for transfection efficiency in all experiments. The plasmid pTZ19, used as carrier DNA, was provided by Dr. Byron Kemper of the University of Illinois.

ER mutagenesis and expression of mutant receptors in cells

S554fs, L540Q, and ER1–530 were generated as previously described (21). The M7 mutant K520D/G521V/E523R/H524L was described previously (17). Complementary DNAs encoding the N-terminal-truncated (ΔA/B) versions of these mutants were generated by replacing the *Hin*dIII fragment of these full-length mutants with the *Hin*dIII fragment of CMV-ΔA/B hER [which deletes nucleotides from the CMV-5 polylinker (22) to codon 176]. The resultant expression vectors contain the human ER-coding region from amino acids 176–595 and produce human ER derivatives that are deleted of residues N-terminal to Met^{176} in the ER primary sequence. Although we could not accurately determine levels of expression in MCF-7 cells for the mutant receptors (ΔA/B dominant negative ERs, S554fs, L540Q, ER1–530, and M7) because of the small percentage of cells transfected and because many of these receptors are indistinguishable on Western blots from endogenous MCF-7 ER, we did compare expression levels in CHO cells. We found comparable levels of these receptors made when equal amounts of expression plasmids were transfected (as reported in Refs. 17, 21, and 23, where expression levels for many of these mutants were determined). We also observed that the ΔA/B dominant negative ERs and ΔA/B wild-type ER were expressed at similar levels after transfection into MCF-7 cells.

Cell culture and transient transfections

MCF-7 human breast cancer cells were maintained in Eagle's Minimum Essential Medium (MEM; Gibco, Grand Island, NY) supplemented with 5% calf serum (Hyclone, Logan, UT), 25 μg/ml gentamycin, 100 U/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco). Before the experiments, cells were maintained for 1 week in MEM containing the above antibiotics and 5% charcoal dextran-treated calf serum (CDCS); they were then cultured for 1 week in phenol red-free MEM with 5% CDCS and the same antibiotics. Transient transfections were performed as follows. Cells were plated at about 4×10^6 cells/100-mm dish, maintained at 37 C in a humidified 5% CO_2 atmosphere for roughly 48 h, and transfected by the $CaPO_4$ coprecipitation method (24). In transactivation assays, 100-mm plates were treated with 1.0 ml DNA precipitate containing 2.0 μg reporter plasmid, 3.0 μg pCH110 internal control plasmid, 0.2–10 μg ER or ER mutant expression vector, and up to 9 μg pTZ carrier DNA. In all cases, cells remained in contact with the precipitate for 4–6 h and were then subjected to a 3-min glycerol shock (25% in MEM plus 5% CDCS). Plates were rinsed, given fresh medium, and treated with E_2, TOT, E_2 plus IBMX/CT, or ethanol vehicle as appropriate. Cells were harvested after 24 h, and extracts were prepared in 250 μl 250 mM Tris, pH 7.5, using three freeze-thaw cycles. β-Galactosidase activity was measured (25) to normalize for transfection efficiency among plates. CAT assays were performed as previously described (26).

Results

ER mutants S554fs and L540Q are potent repressors of E_2-stimulated endogenous ER activity

Three ER mutants were selected for study because they had previously exhibited strong dominant negative activity in transfected CHO cells (17). The mutants, generated by random chemical mutagenesis, include a frame shift (S554fs), a point mutation (L540Q), and a truncated receptor (ER1–530) (21). MCF-7 cells were transfected with either the ERE-tk-CAT or $(ERE)_2$-pS2-CAT reporter plasmid in addition to expression vector for the ER mutant under examination. CAT activity in response to a saturating dose of E_2 (10^{-9} M) was then measured for each mutant studied. The data in Fig. 1 indicate dramatic differences in resultant CAT activity between MCF-7 cells into which no ER mutants were introduced and those transfected with dominant negative ERs. Whereas endogenous MCF-7 ER exhibited a 70-fold induction of transcriptional activity (set at 100%) from an ERE-tk-CAT reporter in response to 10^{-9} M E_2, cells transfected with 10 μg expression vector for S554fs, L540Q, and ER1–530 exhibited 87%, 97%, and 62% repressions of E_2-induced transcription, respectively (Fig. 1). Lesser amounts of expression vector for each mutant were used in an attempt to gauge their relative potencies as dominant negative inhibitors. These studies showed S554fs to be the most potent of the three ER mutants in inhibiting E_2-induced transcriptional activity in MCF-7 cells (Fig. 1). When a reporter gene containing the pS2 promoter, $(ERE)_2$-pS2-CAT, was used in similar experiments, E_2 stimulated a 30-fold increase in MCF-7 ER transcriptional activity, and 10 μg expression vector for S554fs, L540Q, and ER1–530 repressed ER-mediated transcription by 90%, 97%, and 85%, respectively (Fig. 2). Comparative studies with lesser amounts of the three mutants again showed S554fs to be the most potent of the three. Another ER mutant, K520D/G521V/E523R/H524L (M7), which was previously determined to be transcriptionally inactive and to show only modest ER inhibitory activity in CHO cells (17, 27), was assayed for dominant negative activity in the MCF-7 cell system. Consistent with its weak dominant negative activity in CHO cells, the M7 mutant failed to inhibit ER-mediated transcription from either reporter gene employed in this study when transfected at the 1.5 μg expression plasmid level (Figs. 1 and 2), whereas it demonstrated some suppressive activity at the 10-μg plasmid concentration, but always much less than that of the three dominant negative mutants. Transfection of 10 μg of the empty vector pCMV5 had no effect on endogenous MCF-7 ER activity (data not shown).

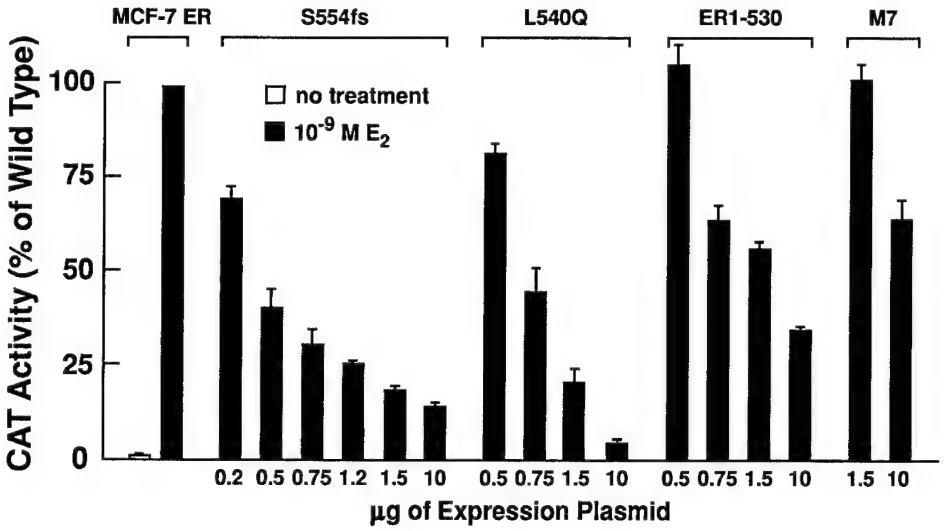

FIG. 1. Dose-response analysis of the ability of ER mutants to block E_2-stimulated transcriptional activity of endogenous ER. MCF-7 cells were cotransfected with the reporter plasmid ERE-tk-CAT; the indicated amounts of expression vector for the ER mutants S554fs, L540Q, ER1–530, and M7; and a β-galactosidase internal reporter to correct for transfection efficiency. Two tenths to 10 µg mutant ER expression vector were employed. Cells were treated with 10^{-9} M E_2 for 24 h. Extracts were prepared and analyzed for β-galactosidase and CAT activity as described in *Materials and Methods*. The magnitude of wild-type (MCF-7) ER activation by E_2 alone was set at 100%. *Error bars* represent the range (n = 2 experiments) or SEM (n = 3–6 experiments). Each value from an experiment is the average of duplicate determinations from two plates of cells.

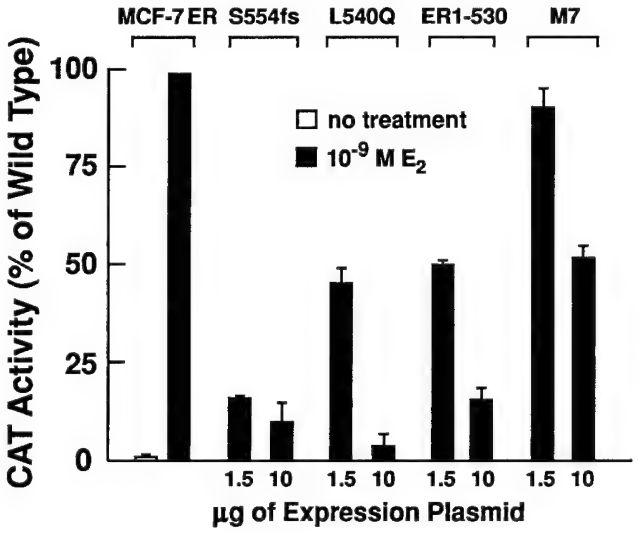

FIG. 2. Examination of the ability of ER mutants to block E_2-stimulated endogenous ER transcriptional activity from a reporter plasmid containing the pS2 promoter. MCF-7 cells were cotransfected with the reporter plasmid $(ERE)_2$-pS2-CAT; 1.5 or 10 µg expression vector for the ER mutants S554fs, L540Q, ER1–530, and M7; and a β-galactosidase internal reporter to correct for transfection efficiency. Cells were treated with 10^{-9} M E_2 for 24 h. Extracts were prepared and analyzed for β-galactosidase and CAT activity as described in *Materials and Methods*. The magnitude of wild-type ER activation by E_2 alone was set at 100%. *Error bars* represent the range (n = 2 experiments) or SEM (n = 3–6 experiments). Each value from an experiment is the average of duplicate determinations from two plates of cells.

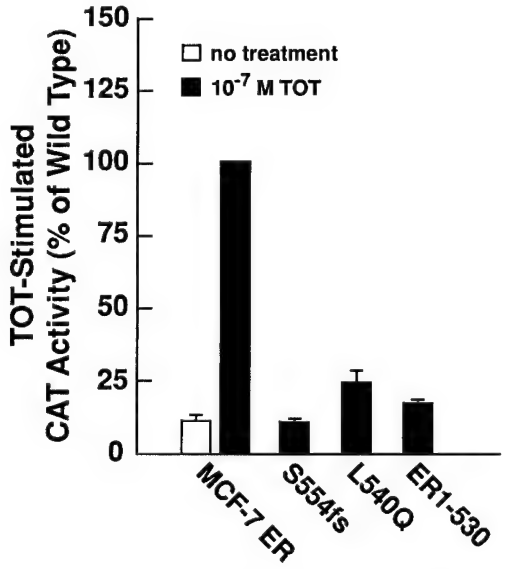

FIG. 3. Examination of the ability of ER mutants to block TOT-stimulated transcriptional activity of endogenous ER. MCF-7 cells were cotransfected with the $(ERE)_2$-pS2-CAT reporter plasmid; 0.2 µg expression vector for the ER mutants S554fs, L540Q, and ER1–530; and a β-galactosidase internal reporter to correct for transfection efficiency. Cells were treated with 10^{-7} M TOT for 24 h. Extracts were prepared and analyzed for β-galactosidase and CAT activity as described in *Materials and Methods*. The magnitude of wild-type ER activation by TOT alone (8-fold) was set at 100%. *Error bars* represent the range (n = 2 experiments) or SEM (n = 3 experiments). Each value from an experiment is the average of duplicate determinations from two plates of cells.

Dominant negative mutants strongly antagonize tamoxifen-stimulated transcription

We next examined whether the ER mutants were capable of inhibiting TOT-stimulated transcription. TOT treatment of MCF-7 cells resulted in a 9-fold induction of ER-mediated transcription, *i.e.* a response about 30% of that elicited by E_2 (Fig. 3). This activity was almost completely eliminated in cells containing any of the transfected dominant negative mutants. A low amount (0.2 µg) of expression vector for S554fs, L540Q, and ER1–530 suppressed 100%, 84%, and 93% of TOT-stimulated transcription, respectively (Fig. 3). Thus, the stimulatory activity of the TOT-occupied MCF-7 ERs appeared to be even more effectively suppressed by the dominant negative ER mutants than was that of the E_2-occupied receptors.

S554fs and L540Q function well as dominant negative receptors in the presence of elevated intracellular cAMP

Recent reports have documented the ability of protein kinase A activators to increase ligand-stimulated transactivation by steroid receptors, including ER (18, 23, 28–32). As such, the ability of the mutant ERs to antagonize ER-mediated transcription in the presence of high levels of intracellular cAMP was assessed by treating transfected MCF-7 cells not only with E_2, but also with IBMX/CT, agents that have been shown to elevate intracellular cAMP in these cells (33). Although there was a strong induction of ER-mediated transcriptional activity from the ERE-tk-CAT reporter gene in response to E_2 treatment (set at 100%), this was elevated consistently (~1.4-fold) when IBMX/CT was also administered to transfected cells. Exposure to IBMX/CT alone had little effect on MCF-7 ER activity. When 0.75 μg expression plasmid for each of the dominant negative mutants was introduced into E_2- plus IBMX/CT-treated MCF-7 cells, S554fs, L540Q, and ER1–530 achieved repressions of 87%, 88%, and 61%, respectively (Fig. 4A, ▨). These levels of inhibition compare favorably to those achieved in the absence of elevated intracellular cAMP and were, in fact, slightly greater. Similar experiments (Fig. 4B) were conducted using the $(ERE)_2$-pS2-CAT reporter gene; E_2 plus IBMX/CT exposure elicited a stimulation of MCF-7 ER CAT activity 2.2-fold that evoked by E_2 alone. Once again, repression of ER activity by the dominant negative mutants in the absence of increased levels of intracellular cAMP was almost identical to that in the presence of added IBMX/CT (Fig. 4B). The experiments suggest that the presence of high levels of cAMP does not impair the ability of these mutants to act as strong dominant negative inhibitors of ER action despite the cAMP-stimulated enhancement of ER transcriptional activity.

ER mutants deleted of their N-terminal transactivation function lose the dominant negative phenotype

The dominant negative ER mutants contain the entire A/B regions of the receptor and, therefore, have an intact N-terminal transactivation (AF-1) domain. These AF-1 regions, which are widely thought to be hormone independent (34), might confer upon the mutants some intrinsic ability to activate transcription, thereby reducing their dominant negative inhibitory action. In an attempt to further increase the potency of the ER mutants as dominant negative ER inhibitors, we deleted the first 175 residues at their N-terminals and, therefore, removed their AF-1 transactivation functions. We then transfected MCF-7 cells with these truncated ER mutants and compared their abilities to function as dominant negative ER repressors with those of the full-length dominant negative mutants. Although 0.5 μg expression vector for S554fs and L540Q achieved 60% and 20% repression of transcriptional activity, and 1.5 μg expression vector for S554fs and L540Q achieved 80–85% repression of transcriptional activity, equivalent amounts of ΔA/B S554fs and ΔA/B L540Q showed little ability to repress E_2 action (Fig. 5). The ER1–530 mutant, although the least effective of the three dominant negative receptors, also became less effective in suppressing endogenous ER activity when present in the truncated (ΔA/B) form (Fig. 5). Using 1.5 μg expression vector, the ER1–530 mutant achieved a 45% repression of endogenous ER activity; the repression was reduced to 15% for the ΔA/B ER1–530 mutant. As such, deletion of the AF-1 transactivation domain from these ER mutants not only failed to increase their potency as dominant negative ER repressors, but it also destroyed their ability to function as effective inhibitors of ER action.

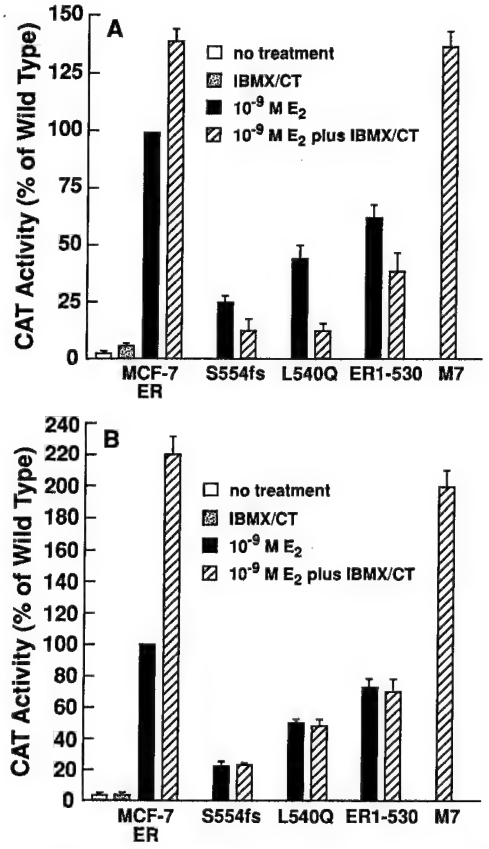

FIG. 4. Examination of the ability of ER mutants to block E_2-stimulated transcriptional activity of endogenous ER in the presence of elevated intracellular cAMP. MCF-7 cells were cotransfected with the ERE-tk-CAT reporter plasmid; 0.75 μg expression vector for the ER mutants S554fs, L540Q, ER1–530, and M7; and a β-galactosidase internal reporter to correct for transfection efficiency (A) or the ERE_2-pS2-CAT reporter plasmid, 1.5 μg mutant ER expression vector, and a β-galactosidase internal reporter (B). Cells were treated with IBMX/CT alone, E_2 alone, or 10^{-9} M E_2 and 10^{-4} M IBMX plus 1 μg/ml CT for 24 h. Extracts were prepared and analyzed for β-galactosidase and CAT activities as described in *Materials and Methods*. The magnitude of wild-type ER activation by E_2 alone was set at 100%, and all values (with and without IBMX/CT exposure) are expressed as a percentage of the value for wild-type ER plus E_2 alone. *Error bars* represent the range (n = 2 experiments) or SEM (n = 3 experiments). Each value from an experiment is the average of duplicate determinations from two plates of cells.

Discussion

We report that two human ER mutants, S554fs and L540Q, are potent dominant negative inhibitors of endogenous ER transcriptional activity in MCF-7 human breast cancer cells. A third mutant, ER1–530, is a weaker repres-

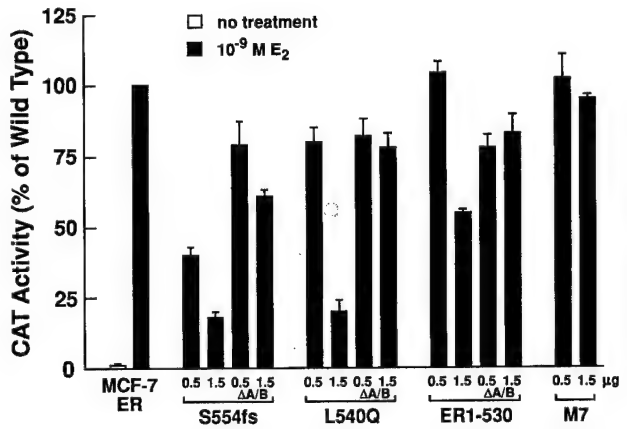

FIG. 5. Examination of the ability of ΔA/B ER mutants to block E_2-stimulated transcriptional activity of endogenous ER. MCF-7 cells were cotransfected with the ERE-tk-CAT reporter plasmid, a β-galactosidase internal reporter to correct for transfection efficiency, and 0.5 or 1.5 μg expression vector for the ER mutants S554fs, ΔA/B S554fs, L540Q, ΔA/B L540Q, ER1–530, ΔA/B ER1–530, and M7. Cells were treated with 10^{-9} M E_2 for 24 h. Extracts were prepared and analyzed for β-galactosidase and CAT activities as described in *Materials and Methods*. The magnitude of wild-type ER activation by E_2 alone was set at 100%. *Error bars* represent the range (n = 2 experiments) or SEM (n = 3 experiments). Each value from an experiment is the average of duplicate determinations from two plates of cells.

sor of ER action in this cell line. As S554fs has previously been shown to bind to ERE DNA with a lower affinity than that of wild-type ER (17), its relatively high potency as a dominant negative ER in MCF-7 cells may arise from an ability to form heterodimers with the wild-type ER, which are transcriptionally compromised. Alternatively, it could be the result of a greater ability on the part of S554fs to sequester cellular factors with which wild-type ER interacts to activate transcription. Transcriptional inactivity alone is not sufficient to confer a strong dominant negative phenotype, however, because the ER mutant M7 was not an effective repressor of MCF-7 ER activity at concentrations (0.5 or 1.5 μg) at which the dominant negative ER mutants showed suppressive activity. At higher plasmid concentrations (10 μg), M7 showed some suppressive activity, consistent with its ability to act as an ER-selective inhibitor at high concentrations (17, 27).

S554fs, L540Q, and ER1–530 all proved to be extremely effective inhibitors of TOT-stimulated ER activity. It is possible that the conformation of wild-type ER when bound by TOT (6, 10, 35) may lend the receptor to easy suppression not only by S554fs and L540Q, but also by ER1–530.

Given reports documenting the ability of protein kinase A activators to increase ligand-stimulated transactivation by ER (23, 28) as well as recent studies by us demonstrating the occasional transcriptional activation of the ER mutants S554fs and L540Q in some cell and promoter contexts by a combination of estrogen or antiestrogen ligands and agents that elevate intracellular cAMP (18), we assessed the ability of the mutant ERs to antagonize ER-mediated transcription in the presence of high levels of intracellular cAMP. When the dominant negative ER mutants were introduced into E_2- and IBMX/CT-treated MCF-7 cells, S554fs, L540Q, and ER1–530 achieved repressions of 87%, 88%, and 61%, respectively, which compare favorably with those achieved in the absence of elevated intracellular cAMP. As it is now clear that cell and promoter context markedly influence transcriptional activation by the ER (34, 36) and other steroid and thyroid hormone receptors (37, 38), it is possible that elevated levels of cAMP in MCF-7 cells modulate either the conformation or the activity of wild-type ER, the mutant ERs, or cellular factors with which they interact, so as to maintain or even enhance the dominant negative effects seen.

Of note, we observed that deletion of the N-terminal A/B domain of the dominant negative receptors, which contains the AF-1 transactivation region, rendered them ineffective. Therefore, it appears that the N-terminal region of the ER, which is known to interact with other cellular factors (34, 36), is necessary for the ER mutants to function as dominant negative inhibitors. This raises the distinct possibility that the mutants may need to interact with cellular factors other than the ER to achieve their inhibitory effects and is consistent with the promoter dependence of the dominant negative phenomenon. For example, the mutants, especially ER1–530, differed somewhat in their effectiveness in suppressing MCF-7 ER activity on the estrogen-responsive tk *vs.* pS2 promoter gene constructs studied. On the other hand, the possibility that the N-terminal-truncated ER mutants may be impaired in some other function, such as dimerization, cannot be formally discounted, and experiments exploring these issues are being undertaken.

Recent studies have revealed the presence of ER variants, some demonstrating dominant negative activity, in breast cancers (1). These naturally occurring variants are truncated receptors due to the deletion of exon 3 (39) or exon 7 (40). Their role in modulating the response of wild-type ER to endocrine therapies is an issue of great interest. Our studies indicate that potent dominant negative ER mutants can markedly suppress the activity of the endogenous wild-type ER in breast cancer cells.

In summary, ER mutants S554fs and L540Q seem to be potent repressors of ligand-stimulated transcriptional activity in MCF-7 cells. Although cAMP significantly elevates wild-type ER-mediated transcriptional activity, the presence of elevated levels of intracellular cAMP does not seem to thwart the ability of any of these mutants to function as dominant negative ER suppressors in MCF-7 cells; in fact, in these cells, it sometimes appeared to enhance their inhibitory function slightly. The results, taken as a whole, strongly suggest the suitability of these ER mutants for further experiments aimed at suppressing not only the ligand-induced transcriptional activity of ER in MCF-7 human breast cancer cells, but also the stimulation of cell growth and proliferation.

Acknowledgments

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Responses to Pure Antiestrogens (ICI 164384, ICI 182780) in Estrogen-Sensitive and -Resistant Experimental and Clinical Breast Cancer[a]

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INTRODUCTION

The last ten years has seen the emergence of a new class of pharmacological agents termed pure antiestrogens (reviewed in Refs. 1, 2). These compounds, which were originally discovered by ICI Pharmaceuticals Division (now Zeneca Pharmaceuticals) in the UK, have the unique property of binding to the estrogen receptor (ER),[3] producing a receptor complex which lacks estrogenic activity.[4,5] They are of use in two important areas of breast cancer research. Firstly, as clinical agents, where it is hoped that their ability to induce total estrogen deprivation will improve the effectiveness of endocrine therapy. Secondly, as pharmacological probes to investigate the cellular and molecular actions of estrogens and tamoxifen. Inherent in each of these areas of research are questions associated with the impact pure antiestrogens may have on the therapy of endocrine-resistant states and whether resistance develops as a consequence of incomplete estrogen withdrawal; with tumor cells more efficiently utilizing either a reduced estrogenic pool or the agonistic activity of an antiestrogen,

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or whether the resistant cells have completely circumvented the need for ER-mediated growth and hence sensitivity to pure antiestrogens.[2]

Since pure antiestrogens are now entering clinical development, the current paper seeks to outline some of their basic cellular and antitumor properties on estrogen-responsive (MCF-7) human breast cancer cells *in vitro*, primarily using the lead compound ICI 164384. This information will be briefly compared with the properties exhibited by pure antiestrogens in endocrine-resistant variants of human breast cancer cells (see refs. in Ref. 2) and phase I and II trials of ICI 182780 in primary[5,6] and advanced[7] breast cancer patients. Where possible examples will be given from immunohistochemical studies, since this technique is most readily applicable to clinical material and ultimately should facilitate an assessment of the degree to which pure antiestrogens are fulfilling their potential as complete antagonists of estrogen action in clinical breast cancer and thereby aid in defining the importance of estrogens in the regulation of breast cancer growth and development.

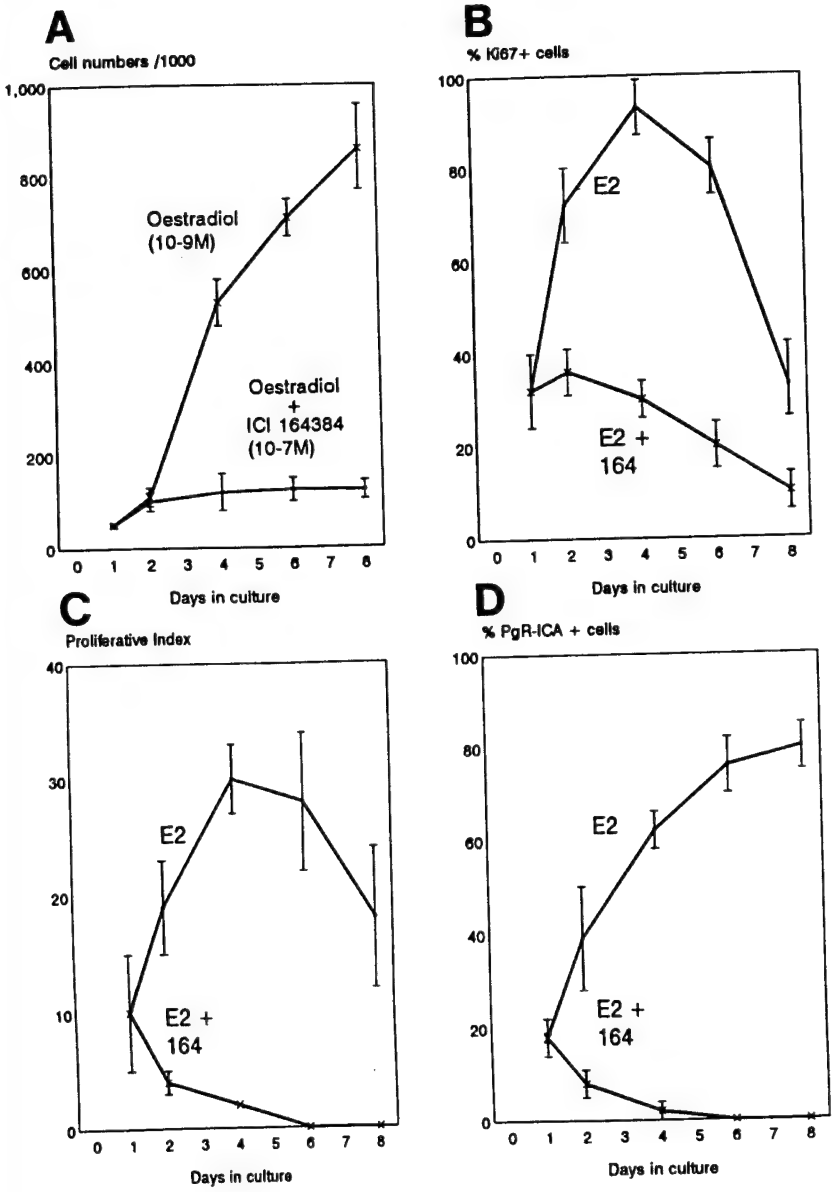
Experimental Studies with Pure Antiestrogens

Biological Consequences of Exposure of Breast Cancer Cells In Vitro *to Pure Antiestrogens*

Evidence from breast cancer cells grown in culture suggests that pure antiestrogens may be highly efficient in counteracting the stimulatory effects exhibited by estrogens both on cell proliferation and on steroid hormone-regulated gene expression.

One of the most important early observations arising from the functional disablement of ER signalling by pure antiestrogens in estrogen-sensitive human breast cancer cell lines was that, in contrast to the stimulatory activity of estradiol, treated cells became efficiently growth arrested (FIG. 1A).[8–10] This action is reflected in the growth dynamics of the tumor cells, with several groups showing that while estradiol increases the tumor cell growth fraction and acts to stimulate the passage of cells through the cell cycle, pure antiestrogens promote a highly effective restriction of the cell cycle approximately 5 hours into G1 and hence cause a reduction of the proportion of cells undergoing DNA synthesis.[8,9] On continuous exposure to pure antiestrogens there is almost a complete loss of those nuclear antigens which mark cell proliferation (FIG. 1B,C)[10] as a large proportion of the cells pass into a noncycling population.[9] Such cells show a reduced RNA/DNA ratio, characteristic of Go (Nicholson, Francis and Hoy, unpublished results). It is noteworthy that the growth-inhibitory activity of pure antiestrogens is not solely restricted to cytostatic activity; on continuous exposure there also appears to be a limited cytotoxic component.[5]

The growth-inhibitory activity of pure antiestrogens on human breast cancer cell lines is characteristically preceded by changes in the expression of several estrogen-regulated genes,[5,10–14] with, for example, the high levels of nuclear immunodetectable PR induced in estradiol-treated MCF-7 cells being rapidly reversed by a 100-fold molar excess of ICI 164384 (FIG. 1D).[5] Indeed, examination of the percentage of PR-positive cells throughout estradiol and ICI 164384 treatment shows that not only does the pure antiestrogen block estradiol-induced PR levels, but that it also obliterates all PR signalling after 4 days of culture. Such cells are as a consequence no longer responsive to progesterone.



Similarly, the substantial increase in cytoplasmic pS2 immunostaining (a protein of unknown function in the breast) that is induced by estradiol in human breast cancer cells is largely absent following ICI 164384 treatment.[5] Any residual pS2 staining tends to be present towards the outer cell membrane in small secretory vesicles. Once secreted, however, the cells remain negative, with no evidence of further pS2 production within the endoplasmic reticulum.

Predictably, the decrease in estrogen-regulated proteins often parallels a highly significant fall in their mRNA levels,[5,11,13,14] with, for example, pS2 mRNA levels being undetectable following 5 days of ICI 164384 treatment.[5] Indeed, even after reverse transcription PCR (30 cycles), the pS2 mRNA has been shown to be barely detectable in ICI 164384- and ICI 182780-treated cells,[5] indicating that pure antiestrogens can produce a rapid and complete shutdown of estrogen-regulated gene function. These actions contrast with the effect of both ICI 164384 and ICI 182780 on the estrogen-suppressed gene sequence pMGT-1, the expression of which is very significantly upregulated in their presence.[15]

A number of the changes in gene expression may directly contribute to the mechanism of action of the drugs, with ICI 182780 promoting decreases in immunodetectable TGFα, an estrogen-inducable mitogenic growth factor,[16] and the bcl-2 protein, a factor which has been implicated in the protection of cells against programmed cell death.[17] In each instance, while these proteins are readily detectable in a high proportion of cells treated by estradiol, their levels are lowered by estrogen withdrawal and further reduced by the pure antiestrogen.[5] This is especially evident for the bcl-2 protein, with estradiol-related immunostaining being largely abolished by ICI 182780. Indeed, bcl-2 positivity is a relatively rare event following the administration of pure antiestrogens and, in line with its role in cell survival, its absence is often associated with the presence of pyknotic tumor cell nuclei.[5]

Comparison with Antiestrogens Exhibiting Partial Estrogen-like Activity In Vitro

The inhibitory actions of pure antiestrogens, initially on estrogen-induced transcriptional events and subsequently on cell proliferation and survival, consistently exceed those effects which may be achieved by established antiestrogens with partial estrogen-like activity.

A comparison of the potency and efficacy of tamoxifen, ICI 164384 and ICI 182780 as inhibitors of the growth of MCF-7 cells showed that the pure antiestrogens are up to two orders of magnitude more potent,[12,21,22] reflecting, in part, their higher

FIGURE 1. Growth and immunohistochemical characterization of MCF-7 cells. The cells were grown in multiwell dishes in white RPMI tissue culture medium with 5% DCC stripped FCS (medium A) containing estradiol ± ICI 164384. **(A)** Cell numbers were assessed using a Coulter Counter; **(B,C)** Ki67 and **(D)** PR assays were performed according to the methods of Bouzubar *et al.*[18] and Press & Greene,[19] respectively. The Ki67 proliferative index (c) was calculated as the proportion of cells showing intense nucleoplasmic and nucleolar staining patterns.[20] The results are shown as the mean ± SD of six replicates.

affinity for estrogen receptors.[3,22] More significantly, flow cytometric analysis of the growth dynamics of the cultured cells showed that, although both classes of agent share the ability to block cell division in the G1 phase of the cell cycle, both ICI 164384 and ICI 182780 were more effective than tamoxifen[9,22] or hydroxyclomiphene[8] in reducing the proportion of cells which remain able to synthesize DNA after prolonged exposure. These activities, which are specific for estrogen receptor signalling,[10] are reflected in the tumor cell growth fraction[5,10] with pure antiestrogens abrogating growth responses to tamoxifen.[9]

These differences observed between pure and partial antiestrogens and the control of tumor cell growth have been ascribed to their interactions with other signalling pathways, with the partial agonistic activity of tamoxifen being amplified by the presence of growth factors.[9] This appears particularly evident for the interaction of tamoxifen and insulin/IGF-1, where a modest growth response to the antiestrogen is considerably increased by the presence of these factors.[9,23] Such activity is much weaker for ICI 164384, with the compound being more effective than tamoxifen in inhibiting the stimulatory activities of IGF-1 and TGFα.

A further feature of the cellular actions of pure antiestrogens which may relate to their improved antitumor activity has recently been revealed by studying their effects on the expression of estrogen receptors.[5,10,24] It has been observed that they are associated with a rapid loss of the receptor protein in estrogen receptor-positive cells, producing after relatively short periods of time cellular estrogen-receptor negativity.[5,10,24,25] This property contrasts with the increases in ER levels that are seen on either estrogen withdrawal or tamoxifen treatment.[5,10] Recent studies by Fawell *et al.*[26] with ICI 164384 have shown that dimerization of the receptor is impaired by the pure antagonist and this may result in the pure antiestrogen receptor complex becoming more fragile and perhaps more sensitive to the normal processes involved in receptor degradation. Certainly, the half-life of the ICI 164384 receptor complex appears substantially shorter[25] than the half-lives of the estrogen receptor and tamoxifen receptor complexes.[26,27]

Comparison with Estrogen Withdrawal In Vitro

Encouragingly and somewhat surprisingly, the effects of pure antiestrogens on the growth of estrogen receptor-positive breast cancer cells substantially surpass the effects of estrogen withdrawal.[10,21] This property has been demonstrated by several groups, with, for example, ICI 164384 severely impairing the growth of MCF-7 cells in phenol red-free medium where the 5% fetal calf serum has been extensively stripped of its endogenous estrogens by charcoal absorption, a procedure which reduces the level of endogenous estradiol to below 10^{-12} M.[5,10] Once again changes in cell numbers correspond to their recorded growth dynamics, with pure antiestrogens decreasing tumor cell S-phase fraction and increasing the proportion of cells in Go/G1 relative to estrogen withdrawn cells.[22]

The local production of hormones by breast cancer cells may play an important role in promoting some cell growth and gene expression in estrogen-withdrawn cells. The cells would potentially be highly sensitive to these, since estrogen receptor levels are elevated following oestrogen withdrawal.[5,10] However, the actions of locally

produced steroids, through the estrogen receptor, would remain vulnerable to the antagonistic activity of pure antiestrogens.

Thus, in charcoal-stripped serum it is possible that some estradiol is formed from polar precursors that are inefficiently adsorbed from the fetal calf serum, the most obvious candidate being estrone sulphate. Indeed, several groups have shown sulphatase activity in estrogen-sensitive human breast cancer cell lines which can initiate the conversion of estrone sulphate to estradiol.[28–30] Such conversions can occur at physiological concentrations of estrone sulphate, generating sufficient quantities of estradiol to stimulate growth in estrogen-withdrawn cells (0.1 nM estrone sulphate significantly increases S-phase fraction). Under these conditions, the nuclear steroids are unconjugated estrone and estradiol.[29] This sulphatase activity is, however, lowered by the presence of ICI 164384,[29,31] with basal PR and pS2 immunostaining abrogated,[5] an effect which appears not to be due to a direct inhibition of the sulphatase enzyme, but rather to be an indirect ER-mediated response.[29]

Furthermore, interactions with other growth signalling pathways potentially amplify the importance of low estradiol concentrations, with several studies demonstrating that the stimulatory actions of estrogens on tumor cell growth and gene expression can be potentiated by EGF/TGFα, IGF-1 and the FGF family.[22,32–34] In the case of EGF/TGFα signalling, this may occur through the activation of EGF-receptor tyrosine kinase activity, which, in its turn, influences key intermediates that interact with ER in the nucleus.[35] This concept is supported by the recent observations that an EGF-specific tyrosine kinase inhibitor[36] can inhibit the expression of PR and pS2 (FIG. 2A,B) in estrogen-withdrawn MCF-7 cells in the presence of maintained amounts of the estrogen receptor and TGFα (FIG. 2C,D), and that similar compounds can inhibit the growth of MCF-7 cells.[37] Importantly, EGF[35] and dopamine[38] have been shown to activate estrogen receptor signalling in the complete absence of its ligand and thus may operate to enhance the constitutive activity of the estrogen receptor in breast cancer cells *in vitro*. The existence of such mechanisms would normally act to protect cells against complete estrogen withdrawal. Importantly, pure antiestrogens have been shown to reduce the above interactions between estrogen receptor and growth factor signalling, generating cells which are desensitized to estrogens, partial antiestrogens, growth factors and dopamine.[23,35,38] Pure antiestrogens diminish estrogen receptor levels and thus reduce the impact of residual estrogens, and also secondary growth factor signalling. Additionally, ICI 182780 has been shown to reduce the intracellular levels of TGFα (FIG. 4B), thereby reducing its potential autocrine stimulation of breast cancer cells. Elevated TGFα immunostaining in estrogen receptor-positive breast cancer has been recently shown to be associated with a decreased sensitivity of the disease to tamoxifen therapy (39). Its reduction therefore in clinical samples might further improve the sensitivity of breast cancer to estrogen withdrawal.

Biological Consequences of Exposure of Tamoxifen or Estrogen-Resistant Breast Cancer Cell Model Systems to Pure Antiestrogens

Initial clinical responses in breast cancer patients to endocrine-type therapies are all too often overtaken by the development of tumor resistance. This is also the case *in vitro*, where cultured human breast cancer cells gradually gain a comparable

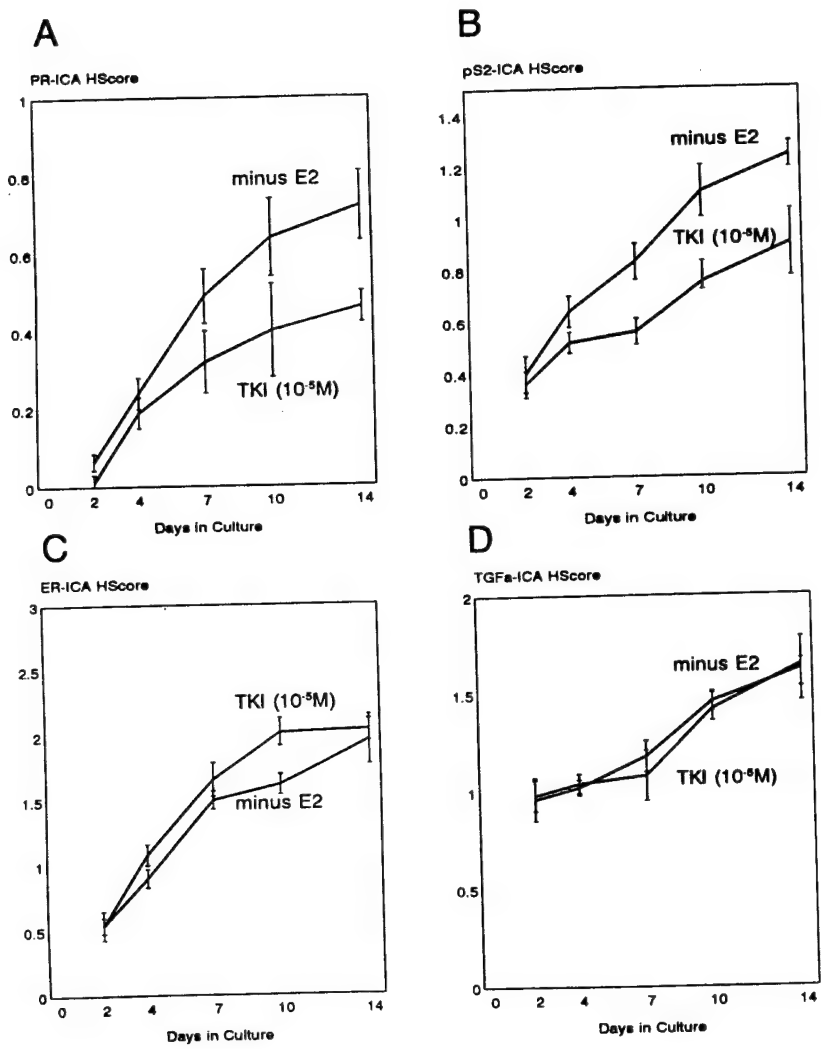


FIGURE 2. Effect of 4-(3-methylanilino)quinazoline (ZM 163613) on MCF-7 cells. The cells were grown on TESPA-coated coverslips in medium A (minus E2) with and without the tyrosine kinase inhibitor (TKI). Assays for PR **(A)**, pS2 **(B)**, ER **(C)** and TGFα **(D)** were performed according to the methods of Press & Greene[19] (A), Charpin *et al.*[50] (B), Walker *et al.*[51] (C) and Nicholson *et al.*[39] (D). The results are the mean values ± SD of 5 replicates from a minimum of 2 coverslips.

endocrine resistance following their prolonged exposure to either estrogen withdrawal or antiestrogens. This phenomenon tends to be associated with an increase in cell growth rate, while retaining the capacity to express estrogen receptors and thus potentially to demonstrate estrogen receptor-mediated responses. Studies with T47D and MCF-7 estrogen-responsive human breast cancer cells suggest that the mechanism underlying the development of tamoxifen resistance[12,40–43] is the outgrowth of subpopulations of cells whose growth is stimulated by tamoxifen,[40,41] rather than the selection of cells which are unaffected by tamoxifen.[44]

Fortunately, it is likely that such tamoxifen-resistant cells retain a sensitivity to the growth-inhibitory actions of pure antiestrogens *in vitro*,[12,13,45] and thus it is feasible that these new compounds may be of clinical value in patients who relapse on tamoxifen therapy. In contrast, cross-resistance to several structurally diverse partial antiestrogens has been demonstrated in the antiestrogen-resistant LY2 cell line.[46,47] A similar resistance mechanism has been identified *in vivo* in animal model systems,[40,47] with tamoxifen-resistant cells again exhibiting growth inhibition by pure antiestrogens.[48,49] This is associated with a parallel decrease in the intracellular levels of estrogen receptors and an associated fall in estrogen-regulated gene products.[49]

Furthermore, it is likely that estrogen-resistant breast cancer cells also retain a sensitivity to the inhibitory actions of pure antiestrogens.[52,53] FIGURE 3A illustrates this phenomenon for a MCF-7 variant cell line, K3.[53–55] This cell line was derived from estrogen-responsive MCF-7 cells by estrogen withdrawal and no longer shows a growth stimulation by estradiol (cmf. response of wild-type cells to oestradiol; FIG. 3B). Despite this, it is growth inhibited by antiestrogens, with ICI 182780 (10^{-7} M) being more effective than 4-hydroxytamoxifen (10^{-7} M). Indeed, only two doublings of the initial cell number occurred throughout ICI 182780 treatment and contrasts with the 7 to 8 doublings recorded under estrogen-treated or -withdrawn conditions. Over several experiments the estimated tumor cell doubling time for ICI 182780-treated K3 cells is in excess of 150 hours. This compares favorably with the value that may be estimated following the treatment of wild-type MCF-7 cells with pure antioestrogens (FIG. 3B).

The actions of ICI 164384 and ICI 182780 cells appear specific for ER signalling and may be reversed by the presence of estradiol.[53] Indeed, K3 cells appear more sensitive to the growth-promoting effects of the steroid than do wild-type cells, with 10^{-9} M and 10^{-8} M estradiol reversing the effects of 10^{-7} M ICI 182780, respectively. As a possible contributor to the increased sensitivity of K3 cells to estradiol, the cells also show a higher basal expression of TGFα (FIG. 4A),[53] which, in contrast to wild-type cells, is not substantially increased by the presence of estradiol.[53] The intracellular concentration of TGFα is, however, lowered by the presence of ICI 182780, but not 4-OHT. Importantly, the reduction in TGFα levels in pure antiestrogen-treated cells accompanies a substantial fall in their ER content (FIG. 4C,D).[53,55] This action would minimize the opportunity for cross-talk between ER and TGFα signalling pathways. Interestingly, K3 cells also show an elevated basal expression of pS2 (FIG. 3C,D),[52] a protein whose gene promoter contains a response element for both estrogens and TGFα.[56] Once again, the expression of this protein is efficiently reduced by the presence of the pure antiestrogen (FIG. 3C,D).[53]

Although less is known about the emergence of breast cancer cells resistant to pure antiestrogens, breast cancer cell xenograft studies have indicated longer remission

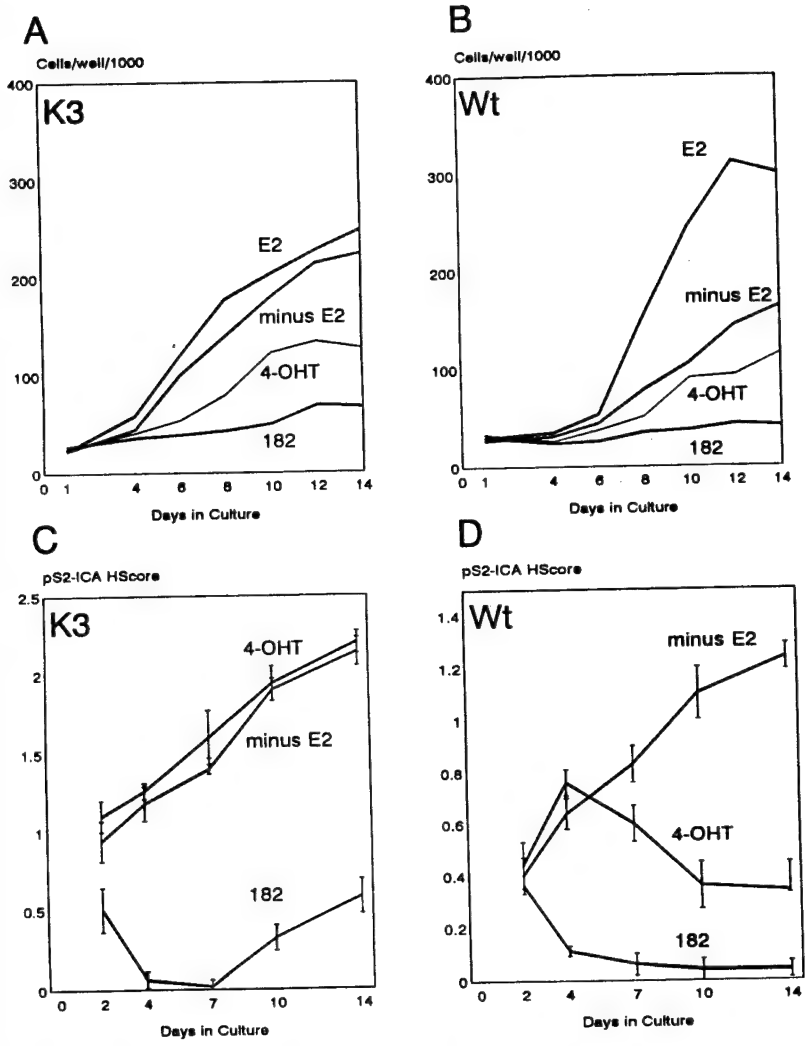


FIGURE 3. Growth and immunohistochemical characterisation of K3 and wild type (Wt) MCF-7 cells. K3 **(A)** and Wt **(B)** cells were grown in multiwell dishes in medium A containing no additives (minus E2), estradiol (10^{-9} M; E2), 4 hydroxytamoxifen (10^{-7} M; 4-OHT) and ICI 182780 (10^{-7} M; 182). Cell numbers were assessed using a Coulter Counter. K3 **(C)** and Wt **(D)** cells were grown on TESPA-coated coverslips in medium A containing no additives, 4-OHT (10^{-7} M) and 182 (10^{-7} M) and assayed for pS2.[50] The results are the mean value ± SD of 5 replicates from a minimum of 2 coverslips.

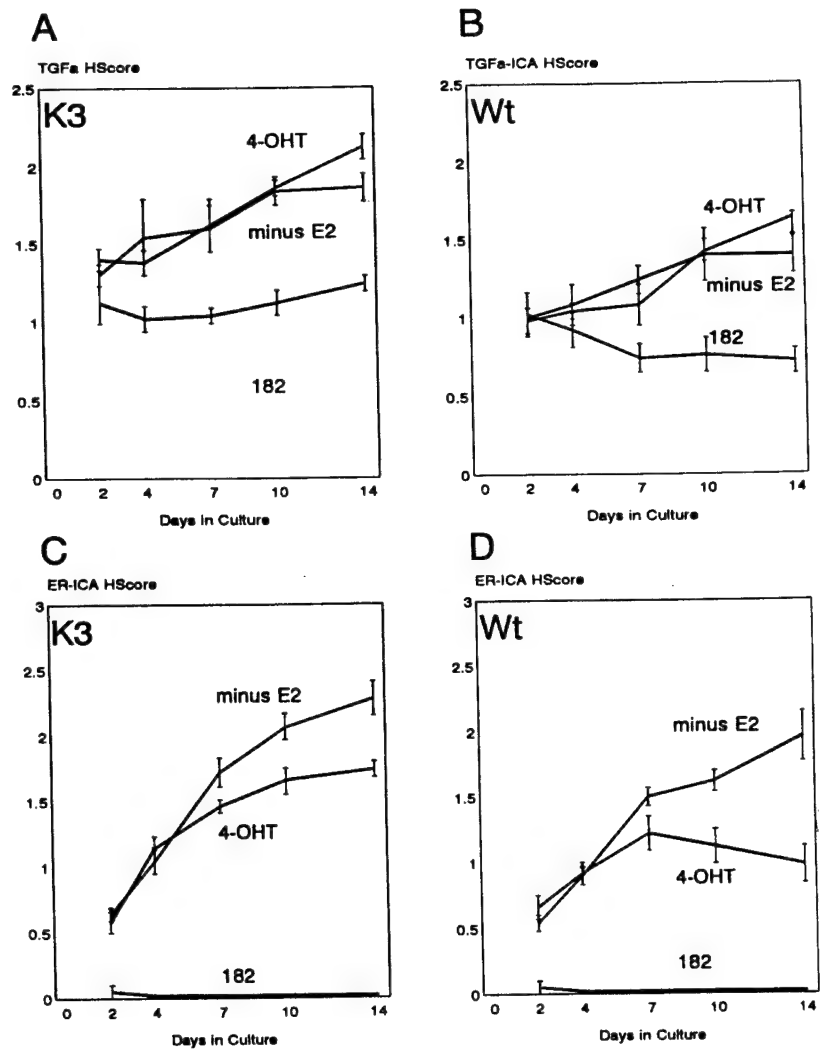


FIGURE 4. Immunohistochemical characterization of K3 and Wt MCF-7 cells. K3 **(A,C)** and Wt **(B,D)** cells were grown on TESPA-coated coverslips under conditions as described in FIGURE 3C,D and assayed for TGFα (A,B) and ER (C,D). The results are the mean value ± SD of 5 replicates from a minimum of 2 coverslips.

times with pure antiestrogens than with either tamoxifen or estrogen withdrawal.[49] Moreover, where ICI 182780-resistant tumors have been transplanted into castrated mice, their growth has been shown to be slightly impeded by tamoxifen alone or in combination with ICI 182780. Such data imply that true cross-resistance to these agents has not fully developed in this animal model, despite the fact that pure antiestrogens abrogate ER levels.[49] Interestingly, this may not be the case *in vitro*, since cultured MCF-7/LCC9 ICI 182780 resistant cells are also tamoxifen resistant.[45]

Properties of Pure Antiestrogens in Clinical Breast Cancer

Antitumor Activity

Although clinical trials with pure antiestrogens are in their infancy and consequently little is known about their clinical properties, in late 1991 a Phase I study of ICI 182780 (6 or 18 mg/day in a short acting propylene glycol-based formulation) was initiated.[5,6] The purpose of this study was to assess the safety and pharmacokinetic properties of the drug and to begin to investigate its biological effects on tumor tissue. The latter was achieved by measuring a number of immunohistochemical end points on pretreatment needle core biopsies from newly diagnosed primary breast cancer patients, comparing results with identical measurements performed on the posttreatment specimen removed at the time of primary surgery seven days later. Analysis of these data has shown that, as observed *in vitro* and within animal model systems, the pure antiestrogen is capable of reducing both tumor cell proliferation and estrogen-regulated gene expression.

In the Phase I study, ICI 182780 produced a highly significant decrease in ER expression in patients with tumors initially classified as ER positive, mirroring those effects observed in experimental systems. Comparison of these data with similar measurements derived from short-term tamoxifen-treated patients showed that although tamoxifen also produced a suppression of ER levels, the effects were not as great as those induced by the highest dose level of ICI 182780 (18 mg/day). Furthermore, examination of PR levels in these samples revealed a divergence of response to the antiestrogens, with the pure antiestrogen inhibiting PR expression while tamoxifen promoted some increase in its tissue concentration. Thus, in initially ER-positive and PR-positive tumors, ICI 182780 caused a fall in PR levels in the majority of patients. Indeed, in several patients the tumors became negative for both of these steroid receptors after the seven-day treatment period. The estrogen-regulated protein pS2 showed similar responses to PR, although the inhibitory effects of ICI 182780 appeared blunted. No short-term effects of ICI 182780 or tamoxifen were recorded on the mammotrophic growth factor TGFα or the bcl-2 protein. However, ICI 182780 (18 mg/day) produced a significant reduction in the expression of the tumor cell proliferation marker Ki-67, with staining values decreasing in the majority of ER-positive tumors examined.

A more recent second study directly attempted to address the issues of whether pure antiestrogens can promote clinical tumor remissions of worthwhile duration and also if these drugs remain effective in tamoxifen-resistant patients.[7] Initial results using an oil-based monthly depot again appear promising. Thus, approximately two-

thirds of women who had either received adjuvant tamoxifen (minimum of two years) and subsequently recurred, or who had relapsed following an initial favorable response to the antiestrogenic drug, gained further benefit from ICI 182780 treatment (7/19 partial responses, 6/19 no change at 6 months). Within responding patients, 10/13 women were still in remission at 9 months. However, although these results are better than would have been expected following tamoxifen withdrawal[57,58] or second line endocrine therapy,[59] the study numbers were small and no direct randomized comparisons were made with other endocrine measures. It is noteworthy, however, that in clinical studies where tamoxifen-resistant tumors have been treated with another triphenylethylene antiestrogen, toremifene, cross-resistance was demonstrated.[56] This result parallels experimental studies on antiestrogen-resistant human breast cancer cells.

Side Effects

To date, no serious drug-related side effects have been reported in either of the above clinical studies. In particular, there has been no evidence of altered coagulation or thrombogenicity after treatment with ICI 182780. On long-term therapy a rise in serum gonadotrophin levels has been recorded, suggesting that ICI 182780 has an antiestrogenic effect on the pituitary gland. There were no significant changes in serum levels of sex hormone binding globulin, implying no estrogenic effect of the drug on the liver.

CONCLUSIONS

It is highly encouraging that the majority of experimental studies to date have shown the recently developed pure antiestrogens to be effective antitumor agents, certainly with regard to inhibition of tumor cell growth and proliferation, and estrogen-regulated gene expression. Indeed, in many instances their effects substantially surpass those observed following estrogen withdrawal or tamoxifen therapy. Treatment of breast cancer cells with pure antiestrogens appears to promote an efficient growth arrest *in vitro* and *in vivo* by their induction of a state of strict estrogen deprivation. Such estrogen withdrawal is likely to be induced primarily by the compounds antagonizing the cellular actions of estrogens and, possibly, reducing other growth signalling activities through the estrogen receptor. Importantly, these mechanisms appear relevant to the treatment of endocrine-resistant states.

In clinical breast cancer it is too early to judge the final value of these compounds. They have, however, passed the first major hurdle by successfully promoting tumor remissions in previously treated patients generating minimal adverse side-effects. The compounds seem capable of reducing cell proliferation and expression of both the estrogen receptor and several estrogen-regulated genes. However, their ultimate success or failure will depend on many factors, most notably the importance of small amounts of estrogen to the tumor cell. If we have not, as yet, passed the threshold of response to estrogen withdrawal, a potential exists for pure antiestrogens to improve the outcome of endocrine therapy[61,62] in such important areas as the rate and duration of remission and the prevention and treatment of resistant states.[2] Over the next few

years, analysis of the clinical actions of pure antiestrogens will establish many unknowns in breast cancer. Let us hope that one of them is that the inhibition of all ER-mediated signalling is a worthwhile goal.[2]

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Estrogen Receptors: Bioactivities and Interactions with Cell Signaling Pathways[1]

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ABSTRACT

Estrogens regulate the growth, differentiation, and functioning of diverse target tissues, both within and outside of the reproductive system. Most of the actions of estrogens appear to be exerted via the estrogen receptor (ER) of target cells, an intracellular receptor that is a member of a large superfamily of proteins that function as ligand-activated transcription factors, regulating the synthesis of specific RNAs and proteins. To understand how the ER discriminates between estrogen ligands, which activate the ER, and antiestrogen ligands, which fail to effectively activate the ER, we have generated and analyzed human estrogen receptors with mutations in the ER hormone binding domain. These studies provide evidence for the promoter-specific and cell-specific actions of the estrogen-occupied and antiestrogen-occupied ER, highlight a regional dissociation of the hormone binding and transcription activation functions in domain E of the receptor, and indicate that some of the contact sites of estrogens and antiestrogens in the ER are likely different. In addition, multiple interactions among different cellular signaling pathways are involved in the regulation of gene expression and cell proliferation by the ER. In several cell types, protein kinase activators and some growth factors enhance the transcriptional activity of the ER. Cyclic AMP also alters the agonist/antagonist balance of some antiestrogens. Estrogens and, to a lesser extent, antiestrogens, as well as protein kinase activators and growth factors increase phosphorylation of the ER and possibly other proteins involved in the ER-specific response pathway, suggesting that changes in cellular phosphorylation state will be important in determining the biological activity of the ER and the effectiveness of antiestrogens as estrogen antagonists. The ER also has important interrelationships with the progesterone receptor (PR) system in modulation of biological responses. Liganded PR-A and PR-B can each suppress estradiol-stimulated ER activity, with the magnitude of repression dependent on the PR isoform, progestin ligand, promoter, and cell type. These findings underscore the mounting evidence for the importance of interactions between members of the steroid hormone receptor family.

OVERVIEW: THE DIVERSITY OF ESTROGEN TARGET TISSUES

The actions of estrogenic hormones are mediated through the estrogen receptor (ER), a member of a large superfamily of nuclear receptors that function as ligand-activated transcription factors. These receptor proteins share a common structural and functional organization, with distinct domains that are responsible for ligand-binding, DNA-binding, and transcription activation [1–5].

Two highly conserved regions are observed in these receptors, one in approximately the middle of the protein (known as domain C), which is involved in interaction with DNA, and one in the carboxy-terminal region (known as domain E/F) that binds hormones and is structurally and functionally complex. Upon binding estrogen, the ER binds to estrogen-response-element DNA, often located in the 5′ flanking region of estrogen responsive genes. These DNA sequences function as enhancers, conferring estrogen inducibility on the genes. The estrogen-occupied receptor is then thought to interact with transcription factors and other components of the transcriptional complex to modulate gene transcription [4–8].

Estrogens, acting via the ER, play important roles in regulating the growth, differentiation, and functioning of many reproductive tissues including the uterus, vagina, ovary, oviduct, and mammary gland. In the uterus and mammary gland, estrogens increase proliferation and alter cell properties via, at least in part, the induction of growth factors and growth factor receptors, an effect largely antagonized by antiestrogens [9–13]. Estrogens also have important sites of action in the pituitary, hypothalamus, and specific brain regions, while exerting crucial actions as well on other tissues including bone, liver, and the cardiovascular system [14–16]. Thus these hormones exert their effects on many, diverse target tissues. Because of this diversity of estrogen target tissues, much current interest focuses on trying to understand the basis for the cell context- and promoter context-dependent actions of estrogens and antiestrogens [17–20] and on the development of estrogens and antiestrogens with enhanced tissue-selective activities.

The actions of estrogens are antagonized by antiestrogens, which bind to the ER in a manner that is competitive with estrogen; but antiestrogens usually fail to effectively activate gene transcription [21–25]. The structures of some estrogens and antiestrogens are shown in Figure 1 and, as can be seen, they include both steroidal and nonsteroidal compounds. Antiestrogens typically have a basic or polar side chain, and this side chain is essential for their antiestrogenic activity. Antiestrogens are of particular interest and utility because of their effectiveness in suppressing the estrogen-stimulated proliferation and metastatic activity of ER-containing breast cancers [9–11, 13, 21–25].

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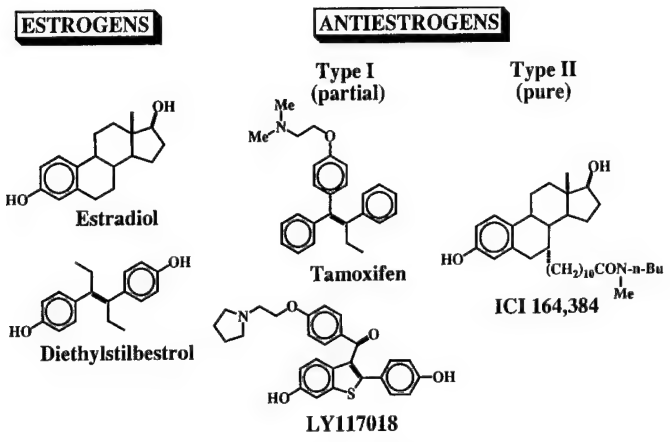

FIG. 1. Structures of several estrogenic and antiestrogenic ligands for the estrogen receptor. The antiestrogens include the nonsteroidal compounds tamoxifen and LY117018 and the steroidal antiestrogen ICI164,384.

ESTROGEN RECEPTOR STRUCTURE-ACTIVITY RELATIONSHIPS

In order to better understand the bioactivities of estrogens and antiestrogens and their differing interactions with the ER, we have focused some of our studies on identifying the regions of the ER that are involved in estrogen and antiestrogen binding and in discriminating between estrogen and antiestrogen [26–31]. Since the hormone-binding domain of the ER is large (more than 250 amino acids), analysis of its structure and its functional complexity is challenging. We have used three approaches for studying estrogen receptor ligand-receptor-response relationships, namely, affinity labeling [32] site-directed mutagenesis, and region-specific chemical mutagenesis of the hormone binding domain.

Many of our studies have analyzed in detail the hormone binding domain of the estrogen receptor, regions E and F, since this domain of the receptor contains both hormone binding and hormone-dependent transactivation functions of the receptor. In our attempts to understand how the receptor discriminates between estrogen and antiestrogen ligands, we have generated and analyzed variant human estrogen receptors with mutations in the ER hormone-binding domain and studied the activity of these receptors on different estrogen-responsive genes in several cell backgrounds when liganded with antiestrogenic or estrogenic ligands. These studies and those of others [17–20] have provided consistent evidence for the promoter-specific and cell-specific actions of the estrogen-occupied and antiestrogen-occupied ER. In addition, although the binding of estrogens and antiestrogens is mutually competitive, studies with ER mutants indicate that some of the contact sites of estrogens and antiestrogens are likely different [29–31, 33]. Our recent studies also reveal that the presence of the carboxy-terminal F domain of the ER is important in the transcription activation and repression activities of antiestrogens and that it affects the magnitude of liganded ER bioactivity in a cell-specific manner [18]. The influence of the F domain on the agonist/antagonist balance and potency of antiestrogens supports its specific modulatory role in the ligand-dependent interaction of ER with components of the transcription complex. These studies ([18, 26–34], see below) have provided evidence for a regional dissociation of the hormone binding and transcription activation regions in domain E of the receptor and have also shown that mutations in the hormone binding domain and deletions of C-terminal regions result in ligand discrimination mutants, that is, receptors that are differentially altered in their ability to bind and/or mediate the actions of estrogens versus antiestrogens.

A variety of studies [17–20, 26–35] have provided strong documentation that the response of genes to estrogen and antiestrogen depend on several important factors: 1) the nature of the estrogen receptor, i.e., whether it is wild type or variant; 2) the ligand; 3) the promoter; and 4) the cell context. The gene response, in addition, can be modulated by cAMP, growth factors, and agents that affect protein kinases and cell phosphorylation [19, 36–40]. These factors, no doubt, account for differences in the relative agonism/antagonism of antiestrogens like tamoxifen on different genes and in different target cells such as those in breast cancer cells versus uterine or bone cells.

Although both estrogens and antiestrogens bind within the hormone binding domain, the association must differ because estrogen binding activates a transcriptional enhancement function, whereas antiestrogens fully or partially fail in this role. Antiestrogens are believed to act in large measure by competing for binding to the ER and altering the conformation of the ER such that the receptor fails to effectively activate gene transcription. In addition, antiestrogens exert antigrowth factor activities via a mechanism that requires ER but is still not fully understood ([41–43] and refs. therein). Models of antiestrogen action at the molecular level are beginning to emerge, and recent biological studies as well indicate that antiestrogens fall into at least two distinct categories: antiestrogens such as tamoxifen that are mixed or partial agonists/antagonists (type I) and compounds such as ICI164,384 that are complete/pure antagonists (type II). The type I antihormone-ER complexes appear to bind as dimers to estrogen response elements; there, they block hormone-dependent transcription activation mediated by region E of the receptor, but they are believed to have little or no effect on the hormone-independent transcription activation function located in region A/B of the receptor [17]. Thus, they are generally partial or mixed agonist/antagonists, and their action must involve some subtle difference in ligand-receptor interaction, very likely associated with the basic or polar side chain that characterizes the antagonist members of this class. In the case of the more complete antagonists such as ICI164,384, ER conformation must clearly differ from that of the estrogen-occupied ER since some differences in ER binding to DNA and reduction of the ER content of target cells appear to contribute to

[44, 45], but may not fully explain, the pure antagonist character of this antiestrogen [41, 42].

In order to understand how the ER "sees" an antiestrogen as different from an estrogen, we have used site-directed and regional chemical mutagenesis of the ER cDNA to generate estrogen receptors with selected changes in the hormone binding domain. We have been particularly interested in identifying residues in the hormone binding domain important for the binding of estrogen and/or antiestrogen and for the transactivation functions of the receptor, and in elucidating the mechanism by which the ER differently interprets agonistic and antagonistic ligands. Our studies have indicated that selective changes near amino acid 380 and amino acids 520–530 and changes at the C-terminus of the ER result in ER ligand discrimination mutants [18, 26, 29, 30]. These data provide evidence that some contact sites of the receptor with estrogen and antiestrogen differ and that the conformation of the receptor with estrogen and antiestrogen must also be different as a consequence ([29, 33] and refs. therein).

Our observations [26, 31], as well as very important studies by Malcolm Parker and colleagues [34, 46], have shown a separation of the transactivation and hormone-binding functions of the ER with amino acids critical in the transactivation function of the receptor being more C-terminal in domain E (see Fig. 2). Interestingly, some transcriptionally inactive receptors with modifications in this domain E C-terminal activation function 2 (AF-2) region of the ER have potent dominant negative activity, being able to suppress the activity of the wild-type ER in cells [27, 28].

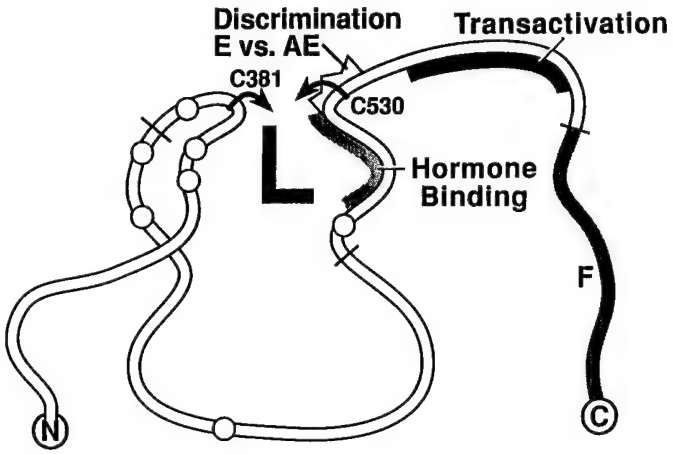

FIG. 2. "Map" of functions in the human estrogen receptor hormone binding domain. Domain E, amino acids 302–553, is shown as is the very C-terminal domain F, amino acids 554–595. Some regions considered to be important in hormone binding, discrimination between estrogen (E) and antiestrogen (AE), and transactivation are highlighted. The ligand (L) is portrayed in a region representing the ligand binding pocket of the receptor. Open circles indicate amino acids in the hormone binding domain where our analyses have shown mutational changes to affect the affinity or stability of hormone binding. See text for description.

ESTROGEN RECEPTOR CROSS TALK WITH OTHER CELL SIGNALING PATHWAYS

We have observed that protein kinase activators enhance the transcriptional activity of the ER and alter the agonist/antagonist balance of some antiestrogens, suggesting that changes in cellular phosphorylation state should be important in determining the biological effectiveness of the estrogen-occupied ER as well as the effectiveness of antiestrogens as estrogen antagonists. Evidence for cross talk between steroid hormone receptors and signal transduction pathways has been increasing. Expression of activator protein (AP)-1, a transcription factor of the *fos*/*jun* heterodimer known to mediate the protein kinase (PK)-C pathway [47], was shown to suppress steroid hormone receptor-mediated gene expression [48], most likely through direct protein-protein interaction between steroid receptors and these oncoproteins [49]. In addition, the ovalbumin gene promoter containing a half-palindromic estrogen-responsive element (ERE) was coactivated by ER and *fos*/*jun* oncoproteins [49–52]. Thus, interaction between these oncoproteins and steroid hormone receptors resulted in cell-specific inhibitory or stimulatory effects on transcriptional activation [50].

Previous studies by us and others [36, 37, 39, 53, 54] documented up-regulation of intracellular progesterone receptor, an estrogen-stimulated protein, by insulin-like growth factor (IGF)-I, epidermal growth factor, phorbol ester, and cAMP in MCF-7 human breast cancer cells and uterine cells. The fact that the stimulation by these diverse agents was blocked by antiestrogen suggested that these agents were presumably acting through the ER pathway [36, 39, 40, 53, 55]. In addition, the fact that protein kinase inhibitors also blocked the effects of estrogen, cAMP, and growth factors suggested the involvement of phosphorylation in these responses. We therefore undertook studies to examine directly whether activators of protein kinases can modulate transcriptional activity of the ER.

In primary cultures of uterine cells, using transient transfection experiments with simple estrogen-responsive reporter genes, we examined the ability of these agents to stimulate ER-mediated gene transcription and also compared the ability of these multiple agents to alter the phosphorylation state of the endogenous uterine ER protein. The results of our study [37] indicate that estrogen, IGF-I, and agents that raise intracellular cAMP are able to stimulate ER-mediated transactivation and ER phosphorylation. The fact that antiestrogen (ICI164,384) evokes a similar increase in ER phosphorylation without a similar increase in transcription activation indicates that an increase in overall ER phosphorylation does not necessarily result in increased transcriptional activity. Also, the observation that transcriptional activation by the ER was nearly completely suppressed by the protein kinase inhibitors H8 and PKI, while the increase in phosphorylation was reduced by 50–75%, indicates that the correlation between transcriptional activation and overall ER phosphorylation is not direct, but it does suggest that some of the effects of estrogen,

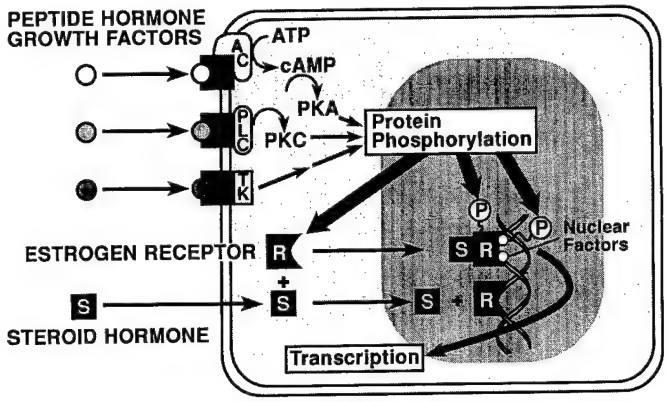

FIG. 3. Model depicting protein kinase-estrogen receptor transcriptional synergism. See text for description.

IGF-I, and cAMP on ER-regulated transactivation are mediated through the activity of protein kinases. Our findings, demonstrating a clear effect of these agents on ER-mediated transactivation, suggest that these agents might also regulate endogenous estrogen target genes, such as that encoding the progesterone receptor, by similar cellular mechanisms.

In order to examine some of the molecular mechanisms controlling transcription of the progesterone receptor gene, we cloned the rat progesterone receptor gene 5′-region and identified two functionally distinct promoters [56]. The two promoters in the rat progesterone receptor gene exhibited differential responsiveness to estradiol and to ER-dependent stimulation by cAMP. The functional differences between these two promoters may lead to altered expression of the A and B progesterone receptor isoforms and, thereby, influence cellular responsiveness to progestins [56].

In MCF-7 human breast cancer cells and other cells, we found that activators of PKA and PKC markedly synergize with estradiol in ER-mediated transcriptional activation and that this transcriptional synergism shows cell- and promoter-specificity [19, 38, 56]. The synergistic stimulation of ER-mediated transcription by estradiol and protein kinase activators did not appear to result from changes in ER content or in the binding affinity of ER for ligand or estrogen response element DNA but, rather, may be a consequence of a stabilization or facilitation of interaction with target components of the transcriptional machinery, possibly either through changes in phosphorylation of ER or other proteins important in ER-mediated transcriptional activation [38]. Of interest also, we have observed that stimulation of the PKA signaling pathway activates the agonist activity of tamoxifen-like but not ICI164,384-like antiestrogens and reduces the effectiveness of tamoxifen as an estrogen antagonist [19]. These findings suggest that agents that enhance intracellular cAMP, such as some growth factors, may contribute to antiestrogen resistance because tamoxifen-like antiestrogens will now be seen by the cell as weak agonists [19, 57]. Related observations have been made with antiprogestins such as RU486 [58–60].

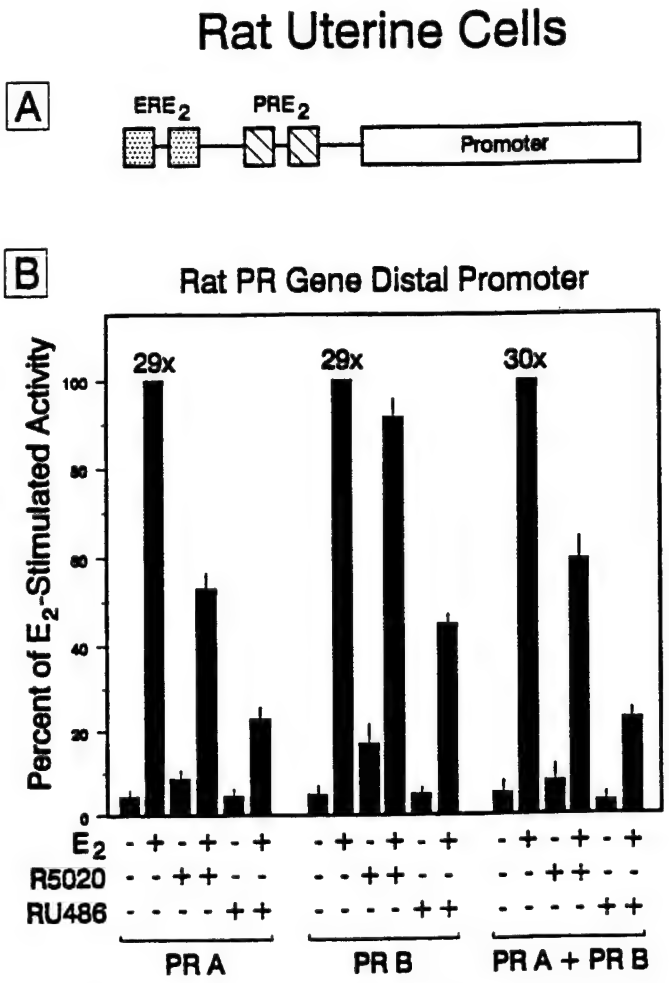

FIG. 4. Repression of ER-mediated transcriptional activity in uterine cells by ligand-occupied progesterone receptors (PRs). **A)** Schematic diagram of the ERE_2PRE_2-Promoter-CAT reporter. **B)** Each 100-mm dish of rat uterine cells was transfected with 500 ng of pRSV-hPRA (labeled PR A), 500 ng of pRSV-hPRB (PR B), or 250 ng each of pRSV-hPRA and pRSV-hPRB (PR A+PR B), in addition to 10 μg of ERE_2PRE_2-PR_{Dist}-CAT, 100 ng of pRSV-rER, and 3 μg of internal control plasmid pCMVβ. The cells were treated with one or more of the following as indicated for 24 h: control vehicle, E_2 (10^{-9} M), R5020 (10^{-8} M), and RU486 (10^{-8} M). The CAT activity in each sample was determined. Each bar represents the mean + SEM for three or more separate determinations. The fold induction in response to E_2 treatment is indicated above the bars. (From Kraus et al. 1995, ref. [68].)

Figure 3 shows a model indicating how we think the protein kinase-estrogen receptor transcriptional synergism might occur. Agents influencing protein kinase pathways may enhance intracellular protein phosphorylation, resulting in either phosphorylation of the ER itself or the phosphorylation of nuclear factors with which the receptor interacts in mediating transcription. Likewise, there is evidence that the steroid hormone itself can alter receptor conformation, increasing the receptor's susceptibility to serve as a substrate for protein kinases [37, 61–64]. There-

fore, agents that increase phosphorylation may, either through phosphorylation of the ER itself or through phosphorylation of nuclear factors required for ER transcription, result in synergistic activation of ER-mediated transcription.

In direct studies on ER phosphorylation, we have shown that estradiol, the antiestrogens trans-hydroxy-tamoxifen and ICI164,384, as well as PKA and PKC activators enhanced overall ER phosphorylation [63]. Tryptic phosphopeptide patterns of wild-type and domain A/B-deleted receptors and site-directed mutagenesis of several serines involved in known protein kinase consensus sequences allowed us to identify serine 104 and/or serine 106 and serine 118—all three being part of a serine-proline motif—as major ER phosphorylation sites. Mutation of these serines to alanines so as to eliminate the possibility of their phosphorylation resulted in an approximately 50% reduction in transactivation activity in response to estradiol while mutation of only one of these serines showed an approximately 15% decrease in transactivation [63]. Of note, estradiol and antiestrogen-occupied estrogen receptors showed virtually identical two-dimensional tryptic phosphopeptide patterns suggesting similar sites of phosphorylation. In contrast, the cAMP-stimulated phosphorylation likely occurs on different phosphorylation sites as indicated by some of our mutational studies [60]; this aspect remains under investigation in our laboratory. Related studies in COS-1 cells by the Chambon laboratory [61] also identified serine 118 as being a major estrogen-regulated phosphorylation site. In MCF-7 cells, the Notides laboratory has also identified serine 118 as a site of ER phosphorylation but has observed serine 167 to be the most prominent site of phosphorylation in these cells [65]. Aurrichio and coworkers [66] have also provided strong evidence for ER phosphorylation on tyrosine 537. The roles of these phosphorylations in the activities (transcriptional and other) of the ER remains an area of great interest.

CROSS TALK BETWEEN ESTROGEN RECEPTOR AND PROGESTERONE RECEPTOR SIGNALING SYSTEMS IN MODULATION OF BIOLOGICAL RESPONSES

In addition to interactions with the signaling pathways described above, the ER also has important interrelationships with the progesterone receptor (PR) system in modulation of responses. This has been well documented biologically in many estrogen target tissues. In the uterus, for example, estrogens increase c-*fos* mRNA, cell proliferation, progesterone receptor mRNA and protein levels, gap junction formation, myometrial contractility, and oxytocin receptors, and these effects are largely antagonized by progesterone ([12, 56, 67, 68] and references therein). The PR is now known to exist as two isoforms in most species, a smaller A form (PR-A) and a larger B form (PR-B); PR-B contains an N-terminal extension of approximately 164

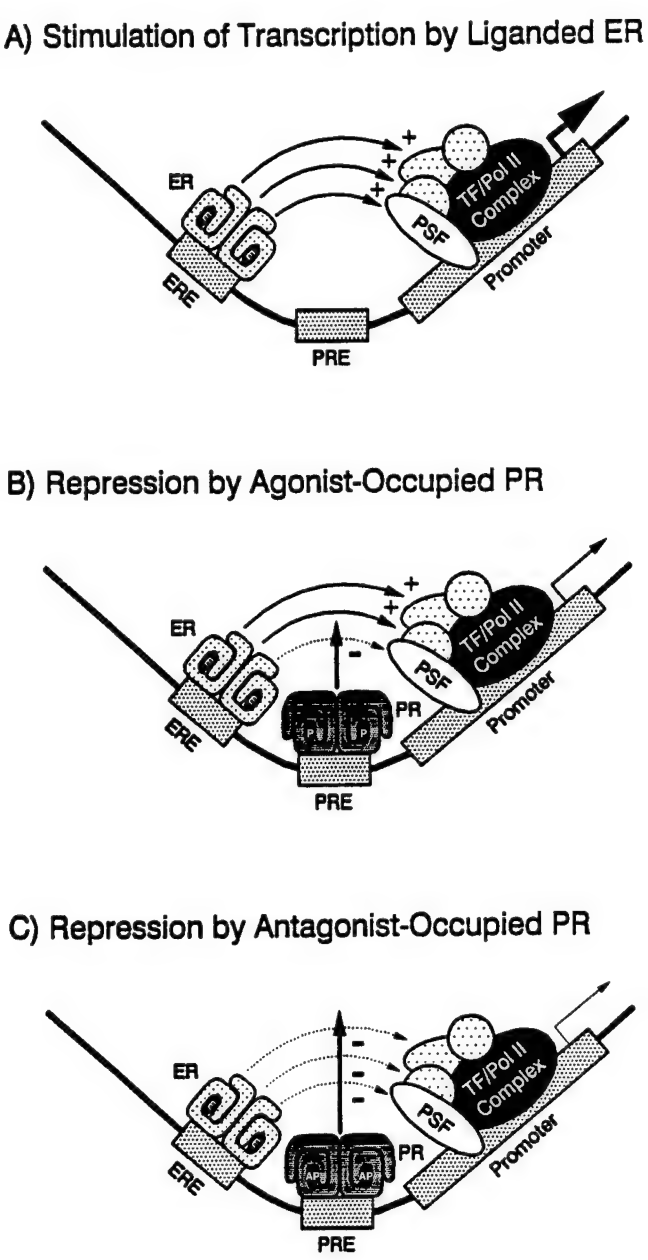

FIG. 5. A model for the repression of ER-mediated transcriptional activity by agonist- and antagonist-occupied PRs. Our findings support a model in which the repression of ER transcriptional activity by liganded PR occurs by quenching. According to this model, liganded PR binds to a site (PRE) distinct from the binding site for ER (ERE) and interferes with the ability of ER to make productive contact with the transcriptional complex. Differences in the magnitude of repression observed for agonist- and antagonist-occupied PRs suggest that agonist-occupied PR only quenches ER-transcription factor interactions that involve the activation function-1 of ER or a promoter-specific component of the ER signaling pathway (PSF), while antagonist-occupied PR quenches a wider range of the ER-transcription factor interactions that occur at the promoter. The individual components of the schematics are labeled. The abbreviations are: AP, antiprogestin; E, estrogen; ER, estrogen receptor; ERE, estrogen response element; P, progestin; PR, progestin receptor; PRE, progestin response element; PSF, promoter-specific factor; TF/Pol II Complex, general transcriptional machinery. (From Kraus et al., 1995, ref. [68]).

amino acids with exact size varying slightly in different species. PR-A and PR-B have differing biological activities on genes [69–71].

In order to understand better how progestins and antiprogestins are able to antagonize the effects of the estradiol-ER complex, we have developed a simplified model system in which estrogen response elements and progestin response elements have been placed upstream of promoters such as the progesterone receptor gene distal promoter, and the effects of PR-A and PR-B alone or together on ER transcriptional activity can be monitored following transfection into uterine cells or other cells in culture [68]. These studies have shown that liganded PR-A and PR-B can each suppress estradiol-stimulated ER activity (Fig. 4) and that the magnitude of repression depends on several factors: the PR isoform (PR-A more effective than PR-B); the progestin ligand (antiprogestin more effective than progestin agonist); the promoter; and the cell type. The effect of cell background is of particular interest since it has been documented that the inhibitory effect of progestin on estrogen action is not equal in all cell types in the uterus [12]. The repression of ER activity by PR in this model system is not due to a reduction of ER levels or to interference with the binding of ER to its response element since PR is still very suppressive even when the progestin response elements are placed more than 2 kb away from the estrogen response elements [68]. Also the fact that PR is antagonistic of ER action at all concentrations of ER studied argues against squelching due to competition for limiting transcription factors.

Our data is most consistent with quenching [72], wherein PR interferes with the ability of ER to interact effectively with the transcription complex, due perhaps to the recruitment of promoter-specific and cell type-specific inhibitory proteins to the promoter (Fig. 5). Related studies by others have also nicely documented PR-A antagonism of ER action [73] as well as the ability of PR-A to suppress the activity of PR-B [71, 74]. These findings underscore the mounting evidence for the importance of interactions between members of the steroid hormone receptor family and begin to address some of the molecular mechanisms underlying these interactions and cross talk.

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MINIREVIEW

Tripartite Steroid Hormone Receptor Pharmacology: Interaction with Multiple Effector Sites as a Basis for the Cell- and Promoter-Specific Action of These Hormones

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INTRODUCTION

The selective action that steroid hormones and the hormones for the other nuclear receptors have in different tissues and on different responses is well known. In fact, this recognized selectivity forms the basis for major efforts, currently underway in the pharmaceutical industry and at universities, toward the development of new, synthetic hormones whose profile of desired activities is optimized for specific therapeutic and preventative applications. This commentary will examine the pharmacological mechanisms that underlie this selectivity.

The study of steroid hormone pharmacology poses particular challenges. *In vivo*, many steroids have pleiotropic activity, displaying a variety of effects in different tissues. Even in cell-based *in vitro* systems, attempts to investigate the molecular basis for steroid hormone action and the selectivity of this action are confounded by the fact that the genomic responses elicited by these ligands can be both primary and secondary (*i.e.* cascade) responses. In the latter situation, the correlation between molecular interaction and response is complex and indirect; this makes it difficult to clearly determine what interactions define the pharmacological parameters of potency and bio-character (biological character, *i.e.* agonist *vs.* antagonist activity) of a specific hormone. Even the genomic actions vary: most involve direct receptor-DNA interaction, but some appear to be mediated via interaction of receptor with other DNA-binding proteins. Steroid hormones may also exert nongenomic effects, some of which may still involve the receptor. In this commentary, we are focusing on the genomic action of steroid hormones that involves the regulation of gene transcription mediated by nuclear receptors.

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THREE MECHANISMS FOR STEROID HORMONE SELECTIVITY

The selectivity that steroid and other hormones for nuclear receptors display at three different levels—the tissue, the cell, and the gene—may be mediated by three distinct mechanisms (Table 1): 1) ligand-based selectivity, 2) receptor-based selectivity, and 3) effector site-based selectivity. Since the first two mechanisms are well recognized, they will be described only briefly; the third mechanism merits careful examination and will be discussed in greater detail.

Ligand-Based Selectivity

By this mechanism, selectivity at the tissue or cell level may be achieved by differences in pharmacokinetics

Table 1. Types of Selectivity in the Action of Ligands for Nuclear Hormone Receptors

Type of selectivity	Components			Level of Selectivity			Mechanism
	Ligand	Receptor	Effector	Tissue	Cell	Gene	
Ligand-based	Different	Same	Same	Yes	Yes	No	Ligand(s) undergoes different metabolism in different tissues/cells (selective bioactivation; selective bioinactivation)
Receptor-based	Same	Different	Same	Yes	Yes	No	Composition of receptors (concentration, subtypes, isoforms, variants) is different in different tissues/cells
Effector-based	Same	Same	Different	Yes	Yes	Yes	The same ligand(s) and same receptor(s) experience different interactions at different effector sites regulating gene transcription

or differential ligand metabolism. The same hormone or set of hormones is presented to different target tissues through the circulation, but their relative amounts within the cell are altered by differential uptake or metabolism—at the level of the target tissue cell. The differential metabolism mechanism may involve either a bioactivation, such as the tissue-selective conversion of the naturally circulating androgen testosterone to the more potent 5α-dihydrotestosterone by the action of 5α-reductase (1), or a bioinactivation, such as the selective oxidation of cortisol, but not aldosterone, by an 11β-dehydrogenase found in tissues that respond to mineralocorticoids (2). Thus, this differential metabolism creates a ligand-based selectivity in which the *same receptor* in different target tissues or cells can experience a *different complexion of hormones* and thereby mediate responses in a selective manner (*cf.* Table 1).

Receptor-Based Selectivity

By the second mechanism, different target tissues experiencing the same hormones may respond in a selective fashion because they have a different composition of receptors. This difference could include variations in the concentrations or ratios of receptor subtypes, isoforms, or splice variants or receptors having different states of covalent modification (*e.g.* phosphorylation) (Refs. 3–5 and references cited therein). This mechanism is especially well represented in the retinoid, thyroid hormone, and vitamin D_3 receptor systems, where multiple receptor forms are found, and different patterns of receptor dimerization are known to be dependent upon both the structure and composition of the ligands and the response elements (6, 7). It appears to be important in the progesterone receptor system, where progesterone receptor A and B isoforms are known to differ in their ability to activate genes (8). Additionally, progesterone receptor A can act as an inhibitor of progesterone receptor B transcriptional activity (9–11). Receptor-based selectivity may also play a role among androgen receptors and glucocorticoid receptors, where two isoforms have been reported (12, 13), and even in some estrogen-responsive cells where full length estrogen receptor and splice variants may coexist (14–18). In these systems, the *same hormone* or set of hormones could effect tissue- or cell-selective action as a result of the *different complexion of receptors* present in different target sites (*cf.* Table 1).

Effector Site-Based Selectivity

Although the former two mechanisms may explain some of the tissue- and cell-selective actions of steroid and related hormones, the selectivity of these hormones clearly also derives from a deeper level. Even in cases where there seems to be no differential hormone metabolism in target tissues and only a single receptor is involved (*i.e.* mechanisms 1 and 2 are not operating), hormones for nuclear receptors are capable of selective action. Most striking is the different biocharacter that some estrogens and their analogs show in terms of certain responses elicited in different target tissues.

For example, in the rat, we have shown that the antiestrogens tamoxifen, nafoxidine, and CI-628 are partial agonists/antagonists in the modulation of pituitary PRL and dopamine turnover in the medial basal hypothalamus (19) and of various responses in the uterus (uterine weight gain, progesterone receptor induction, and plasminogen activator and peroxidase activity stimulation) (20–23), yet they are full agonists in increasing plasma renin substrate in liver (24). In women, raloxifene (originally called keoxifene) shows tissue-selective differences, with strong agonist activity indicated by maintenance of bone density and estrogenic blood lipid profiles, but little stimulation of the uterus (25–30). Tamoxifen therapy in postmenopausal women with breast cancer has also revealed estrogen-like actions of this agent on bone mineral density (31) and lipoprotein levels (32), as well as estrogen-like stimulation of the uterus (33–35), yet little agonism occurs in the breast, where tamoxifen reduces recurrence of breast cancer (36). In contrast, the estradiol-based antiestrogens ICI164,384 and ICI182,780 have almost complete antagonist character in all estrogen target tissues examined, both in experimental cell and

animal systems and in clinical trials in women (37, 38). Regardless of their varying level of agonist or antagonist character in different tissues, these compounds appear to be acting through a single receptor, the estrogen receptor.

The study of the molecular details of steroid hormone pharmacology has been assisted greatly by the development of transient transfection assays, whereby one can achieve independent control over four critical variables, the ligand, the receptor, the gene context, and the cellular milieu. Transfection of estrogen-responsive promoter-reporter constructs into different cells has enabled the regulation of specific genes to be studied in these different cell backgrounds. However, one should keep in mind that hormonal regulation of transfected gene constructs does not always precisely mimic that observed in the native gene context, as local chromatin architecture may be different (39, 40). Nevertheless, the results of these investigations illustrate clearly that *cell-specific factors* can affect the biocharacter (agonist/antagonist balance) of different estrogens.

In studies in several cell types with either wild type or variant estrogen receptors lacking their C-terminal F domains (ΔF), we have observed that the response of these receptors to estrogen and antiestrogen ligands is markedly influenced by cell context (41). For example, in Chinese hamster ovary (CHO) cells and MDA-MB-231 human breast cancer cells expressing wild type or ΔF estrogen receptors, estradiol stimulated equally transcription of several estrogen-responsive promoter reporter gene constructs. By contrast, in HeLa human cervical cancer cells and 3T3 mouse fibroblast cells, the ΔF estrogen receptor exposed to estradiol was much less effective than wild type estrogen receptor in stimulating transcription, and antiestrogens were less potent in suppressing estrogen-stimulated transcription by the ΔF estrogen receptor. These differences in response of the ΔF and wild type estrogen receptor to estrogen or antiestrogen do not appear to be due to a change in receptor expression level, binding affinity for ligands, or binding to estrogen response element DNA. Rather, our data support the supposition that the conformation of the receptor-ligand complex is different with estrogen *vs.* antiestrogen and with wild type *vs.* ΔF estrogen receptor, such that its potential for interaction with protein cofactors or transcription factors is different and is markedly influenced by cell context (41). Likewise, studies by McDonnell and co-workers (42, 43) have provided extensive documentation of the fact that cell background profoundly influences estrogen receptor transcriptional response to ligand. Several groups have shown as well that the transcriptional response of progesterone receptor A and B isoforms to progestin ligands is greatly influenced by the test cell used, as is the ability of progesterone receptor to repress estrogen receptor transcriptional activity (44, 45). This very likely reflects the differing activities of the different activation functions (AF-1, AF-2, and others) in a receptor, a concept nicely documented by Berry *et al.* in 1990 (46) for the estrogen receptor to explain the differing agonist/antagonist activity of tamoxifen in different cells (see below).

Even within the same cell, it is possible to effect selective stimulation of different endogenous genes with different ligands. For example, in estrogen receptor-containing MCF-7 human breast cancer cells, antiestrogens such as tamoxifen are pure antagonists for plasminogen activator activity (47, 48) but show weak agonism for other responses, such as pS2 (39) and progesterone receptor induction (47, 49). By transfecting estrogen-responsive promoter-reporter constructs into these (MCF-7) cells, it has been shown that antiestrogens exhibit *promoter-specific agonism* (50). This promoter-specific agonistic activity of antiestrogens is also observed when these estrogen-responsive promoters are transfected, along with wild type estrogen receptor, into a variety of estrogen receptor-negative cells (41, 42). Further evidence for gene-specific agonist and antagonist properties of tamoxifen and other antiestrogens is evident from studies in GH4 and GC3 pituitary tumor cells, where these compounds act like a full estrogen on some responses yet as an antagonist of estrogen stimulation of other responses (51, 52).

The phenomenon of promoter-specific agonism is particularly well highlighted by the observations made in bone cells with antiestrogens using two different estrogen receptor-dependent responses. Here, raloxifene, a benzothiophene compound typically considered an antiestrogen, tamoxifen, and ICI 164,384 are, in fact, stronger agonists of transforming growth factor-β3 (TGFβ3) promoter activity than estradiol. By contrast, in the same MG-63 osteosarcoma cell cultures, all three ligands act as pure antagonists of the dramatic stimulation of the reporter gene construct estrogen response element-vitellogenin-chloramphenicol acetyl transferase by estradiol (53, 54). Interestingly, the nucleotide sequences comprising the estrogen response elements in these two genes (TGFβ3 and vitellogenin) are quite different, vitellogenin containing a palindromic consensus estrogen response element and TGFβ3 quite a different nucleotide sequence; only the former was shown to bind the estrogen receptor in gel shift assays. The DNA-binding domain of the estrogen receptor appears not to be required for raloxifene induction of the TGFβ3 gene. Since the estrogen receptor does not bind directly to this unusual estrogen response element, an additional DNA-binding protein that tethers estrogen receptor to this enhancer is implied (54). Thus, at least some of the proteins interacting with the ligand-receptor complex at these two promoters would be predicted to be different, to account for the reversed pharmacology displayed by these estrogen receptor ligands at these two genes.

As was mentioned earlier, these findings are also mirrored in tissue-specific differences in the estrogen agonist/antagonist character of these compounds *in*

vivo. Tamoxifen and raloxifene are strong estrogen-like agonists for bone density maintenance in rats and women. They have either some (tamoxifen) or little to no (raloxifene) stimulatory effect on uterine proliferation, yet they are full antagonists of estrogen-stimulated breast cancer cell proliferation and responses such as induction of plasminogen activator activity in breast cancer cells. These observations indicate that these ligands are "selective estrogen receptor modifiers" (27, 30), displaying estrogen agonist or antagonist activity that is dependent on the particular cell and gene endpoint.

Such observations form the basis for efforts currently being directed at the development of tissue-selective estrogen/antiestrogen agents with specific profiles optimal for treatment of women with breast cancer and for postmenopausal bone loss (osteoporosis) prevention: no agonism on breast or uterus; estrogen agonism on bone (for good bone maintenance), the cardiovascular system, and some aspects of liver function (such as blood lipid profile). Such compounds would exploit what is now known about the gene- and cell-selective actions of hormonal ligands and the importance of effector site components in a ligand's pharmacological profile (see below). Thus, in some systems, the same ligand working through a single receptor can elicit a different spectrum of responses from different genes in hormone-responsive cells (*cf.* Table 1). These gene-selective actions cannot be readily explained by either of the first two mechanisms (see above).

EVOLVING MODELS FOR THE ROLE OF THE RECEPTOR IN STEROID HORMONE ACTION—MOLECULAR INTERACTIONS THAT DEFINE POTENCY AND BIOCHARACTER

The Pharmacology of Classical Bipartite (Ligand-Receptor) Systems

The development of the concept of "receptors" in classical pharmacology arose from the need to postulate a molecular species that served as the interface between a drug or hormone and the behavioral or physiological responses that it evoked. The original receptor concept, conceived by Ehrlich (55) and Langley (56), formalized by Clark (57) and Gaddum (58), and refined by Ariëns and Simonis (59) and Stephenson (60) was basically an operational one. Nevertheless, it permitted the different dose-response relationships displayed by various drugs and hormones to be related to a hypothesized molecular interaction that these species had as ligands for the receptor. The activity of these ligands could then be interpreted in terms of the pharmacological parameters "potency" and "biocharacter": potency, measured as the median efficacy (EC_{50}, or median inhibition, IC_{50}), was related to the ligand's affinity for the receptor; biocharacter (*i.e.* agonist *vs.* antagonist character), estimated by the degree to which this binding resulted in activation of the receptor to elicit a response, was related to the ligand's efficacy or intrinsic activity.

At an operational level, the receptor was considered to represent the interface where the molecular interactions with the ligand ceased and the biological responses began. In such a bipartite model, involving only the ligand and the receptor, the ligand plays a role much like that of an allosteric effector of an enzyme, altering the conformation of the receptor and thereby *directly* altering its capacity to elicit the response. The conceptual features of such a bipartite scheme are illustrated in Fig. 1. The key issue is that the receptor itself embodies two functions, the capacity to bind a ligand and the capacity to initiate or effect a response as a direct consequence of that binding. The implications of the bipartite model are subtle but important: since the ligand is controlling the shape and the function of the receptor directly, one can assign to each ligand a unique characteristic potency and biocharacter (Table 2).

The Identification of Steroid Receptors and Their Genomic Action

The preparation of high specific activity radiolabeled steroid hormones more than 3 decades ago led to the identification of specific, high affinity binding proteins in target tissues for steroid hormones (61). Since the binding affinity that these proteins showed for various ligands reflected the biological potency of these ligands quite accurately, the binding proteins were

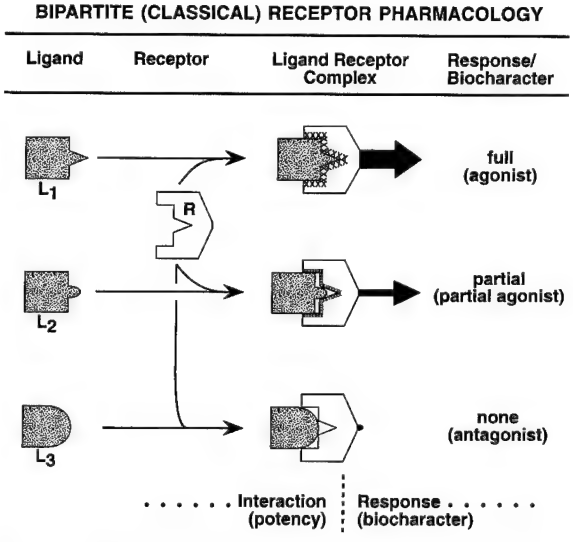

Fig. 1. Classical Bipartite (Ligand-Receptor) Pharmacology

This simple conceptual scheme illustrates how the response to a hormone might be mediated by a bipartite interaction between the hormone, acting as a ligand (L) and a receptor (R). In such a bipartite system, the effect of each hormonal ligand is determined directly by the nature of its interaction with the receptor. Thus, unique potency and biocharacter descriptors can be assigned to each hormone.

Table 2. Pharmacology in Bipartite *vs.* Tripartite Receptor Systems

Pharmacological characteristic	Bipartite scheme	Tripartite scheme
Potency (EC_{50}, IC_{50})	Determined by the affinity of the L-R interaction	Determined by *both* L-R binding affinity and L-R coupling with effectors
Biocharacter (efficacy, intrinsic activity)	Determined by effectiveness the conformation of the L-R complex itself	Determined by *both* shape of the L-R complex and the effectiveness of its coupling with various effector sites
Uniqueness of pharmacological characteristics	Potency and biocharacter can be uniquely assigned to each ligand	Potency and biocharacter are effector dependent; they are not inherent characteristics of a ligand, and cannot be assigned without reference to a particular response

soon referred to as "receptors." Results from other biochemical studies elucidated the principal action of steroids as the activation of gene transcription (for example, Refs. 62–66). The role of these binding proteins as receptors, linking the binding interaction of the steroid with the biochemical response of transcription activation, still appeared to be clear. Nevertheless, it was evident even then that there would be other molecular elements within the cell with which the ligand-receptor complex would need to interact in order for the effect—the transduction of the signal—to continue (67, 68).

In the most recent decade, great strides have been made in determining the structure of these receptors and in elucidating the details of their action. They are multidomain proteins that engage in multiple interactions in the process of eliciting their genetic transcriptional activation or repression responses. In some cases they interact with themselves as homodimers or with other related receptor partners as heterodimers. At each regulated gene, these receptors may interact with DNA via response elements of varying sequence and distribution, with transcription factors and other components of the general transcription apparatus, and with various other activator and adaptor (co-activator and co-repressor) proteins that are associated with the transcriptional regulation of that particular gene (reviewed in Refs. 69–73).

This proliferation of molecular constituents that link ligand to response necessitates a reexamination of the simplistic application of the term "receptor" to this intracellular ligand-binding protein. In fact, *in the nuclear receptor signal transduction cascade, it is no longer so clear where the effect of ligand "interaction" ceases and the biological "response" begins*, and thereby just what molecular entity or entities linking interaction and response merits the appellation "receptor" in the classical pharmacological sense. The "interaction" by which a ligand effects a response in the steroid hormone system is clearly a multipartite phenomenon, one that is much more complex than the bipartite interaction originally envisioned as simply the binding of a hormone to a receptor protein. The proliferation of such terms and phrases as "cell and promoter context," "gene-specific effects," "intracellular receptor pharmacology," "post-receptor events in ligand discrimination," or the "different biology of various receptor-ligand complexes" to describe steroid hormone pharmacology is a reflection of the inadequacy of the current use of the classical terms "agonist," "antagonist, " and "receptor" to describe the selective action of hormones at the level of the cell and gene.

The Tripartite (Ligand-Receptor-Effector) Systems

A tripartite scheme that embodies elements which seem more appropriate to describe steroid hormone molecular pharmacology is shown in Fig. 2 (Table 2). Whereas the bipartite scheme (Fig. 1) embodied the ligand binding and the response initiation functions in one entity, in the tripartite scheme these functions are assigned to separate entities—ligand binding to the receptor, and response initiation to the *effector*. Thus, where there were two partners that defined pharmacology, there are now three: the ligand, the receptor, and the effector.

Tripartite or ligand-receptor-effector schemes were proposed some time ago for certain other signal transduction systems, and more recently even for some glucocorticoid receptor-mediated responses (73a), especially those that showed a discordance between ligand potency in response stimulation (measured as the EC_{50}) and ligand binding to receptor [measured as the dissociation constant (K_d)]. For example in the "spare receptor" hypothesis, the effector was proposed as a response-limiting step beyond the receptor that could account for this potency/binding disjunction (74–77). Many of these systems are now known to be tripartite in reality. For example, the action of extracellular ligands on transmembrane G protein-coupled receptors results in second messenger induction via G protein activation that operates through intracellular sites (78). More recently, the action of immunosuppressants in T cells has been shown to be tripartite; it begins with the binding of the immunosuppressants by immunophilins and then proceeds with the interaction of this complex, as a composite ligand, with the phosphatase calcineurin (79). What is

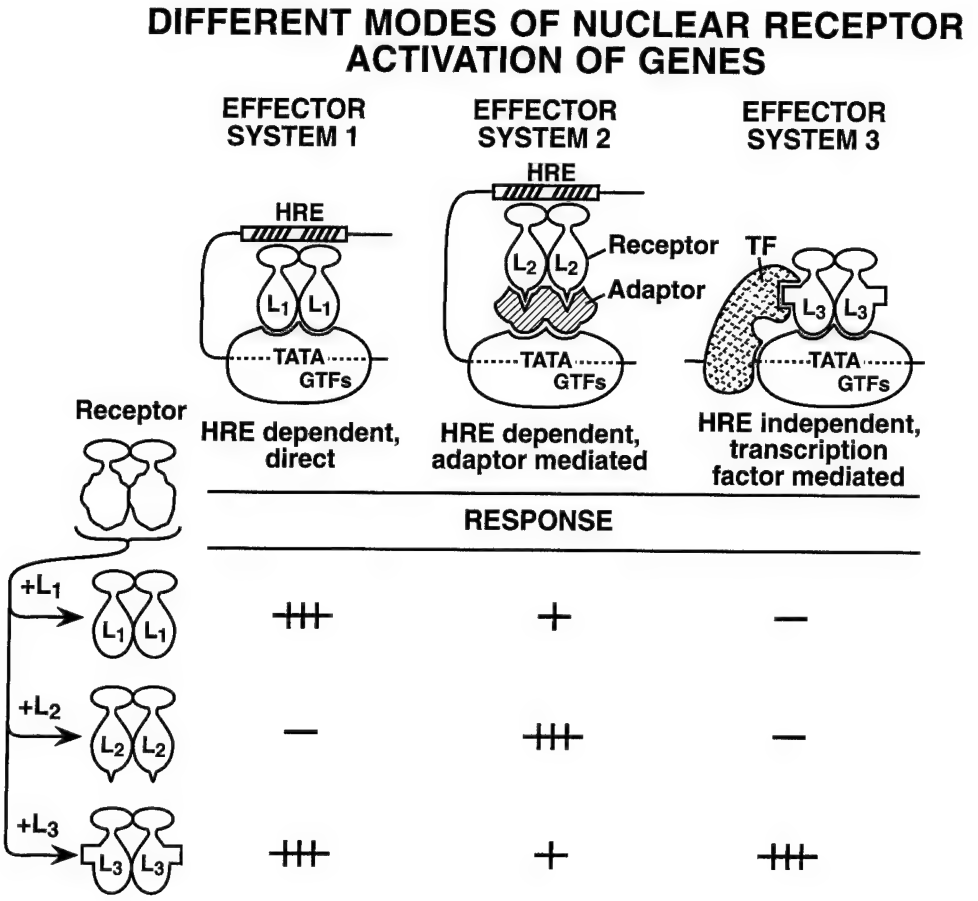

Fig. 2. Tripartite (Ligand-Receptor-Effector) Pharmacology

The response to a hormone is mediated by a tripartite interaction involving the ligand, the receptor, and effector sites through which the ligand-receptor complex regulates the response. The *top* of this scheme illustrates three different modes for nuclear receptor activation of genes; for each mode, an optimal ligand-receptor-effector combination is shown. The *bottom* of the scheme illustrates the activity that each of the three ligand-receptor complexes might have at each of the three effector sites. Note that the receptor adopts a different conformation in its complex with the three ligands and that these different "shapes" affect the nature of the receptor-effector coupling. In a tripartite scheme, the potency of a ligand is determined largely by its affinity of interaction with the receptor, but its biocharacter is determined by the interaction that the ligand-receptor complex has with various effector sites. Therefore, for each receptor, the biocharacter (and to some degree the potency) of a hormone cannot be uniquely assigned without reference to a specific response and effector interaction. Other modes of nuclear receptor gene activation than the three illustrated here, such as the remodeling of nucleosomal and chromatin architecture by hormone receptor complexes, have been identified. However, for simplicity, only three are shown here as examples.

unusual about the tripartite nature of the nuclear hormone receptor system is that there appears to be an unusual number and variety of effectors; this might well be the hallmark of pleiotropic response systems.

The pharmacological implications of the tripartite model are significantly different from the bipartite model. In the bipartite model (Fig. 1 and Table 2), a single interaction, the binding of ligand by receptor, directly regulates receptor function and thereby determines both the potency and the biocharacter of the ligand. By contrast, ligand potency and ligand biocharacter are determined through two different interactions in the tripartite scheme (Fig. 2 and Table 2). In the first interaction, ligand binds to receptor to form a complex, and the affinity of this *binding* is a principal determinant of ligand potency. However, this ligand-receptor interaction alone does not control the response and therefore is not a direct determinant of ligand biocharacter. The pharmacological nature of the ligand, its biocharacter and its potency, is only fully established through the second interaction. This *coupling*, which occurs between the ligand-receptor complex and the third partner, the effector, is an interaction that has both an affinity and an efficacy dimension.

The Nature of Effectors for Nuclear Receptors

In the nuclear hormone receptor systems, the effector site represents the aggregate of all the other components with which the ligand-receptor complex interacts at each regulated gene. Thus, the effector is obviously complex. It is made up of elements common

to all genes, as well as elements unique to each cell and to each gene, even in systems like the estrogen receptor where only a single receptor exists. The nuclear components that define effector-site selectivity are not well understood at present. Nevertheless, they may be grouped into several classes, three of which are illustrated in Fig. 2.

In most cases, the coupling between the receptor and effector involves direct interaction with DNA through hormone response elements, which at various genes may be consensus, nonconsensus, single, multiple, half-sites, etc.; DNA sequences flanking the response elements, which are known to affect receptor binding affinity, also differ in various responsive genes. For the most part, sequences that bind receptors with high affinity act as tethering sites for these potent gene activators. In certain instances such as the proliferin gene, upstream binding to a specific sequence appears to favor a conformationally inactive form of the glucocorticoid receptor and may be operationally defined as a negative glucocorticoid response element (80).

After binding to their cognate response elements, a number of receptors appear to touch the general transcription factor complex (GTFs) located at the TATA box (*cf.* Fig. 2, effector system 1) (81–83). Although TFIID may be a target for certain receptors, the preferred partner of progesterone, estrogen, thyroid hormone, vitamin D_3 receptors, and COUP-TF often appears to be TFIIB, a rate-limiting component whose presence appears requisite for drawing RNA polymerase (and TFIIF) to the promoter (84). At this level, both positive and negative associations have been predicted for receptors with TFIIB. For example, unoccupied thyroid hormone receptor touches TFIIB at two distinct regions; one of these interactions has been hypothesized to be repressive, to explain the well described silencing activity of ligand-free thyroid hormone receptor at certain genes (82). Thyroid hormone binding to thyroid hormone receptor inhibits this repressive interaction. Nevertheless, effector site interactions appear to be of even greater complexity.

Experimental evidence has predicted the existence of adaptor proteins that may act as either coactivators (85, 86) or corepressors for nuclear receptors (*cf.* Fig. 2, effector system 2). In transfected cells, the ability of activated estrogen receptor to suppress or "squelch" the transcriptional capacity of activated progesterone receptor has been interpreted to result from their competitive interactions with limiting concentrations of a putative cellular coactivator (87–89). Recently, this hypothesis has been substantiated by the identification and cloning of a general steroid receptor coactivator (SRC-1), which fulfills many of the criteria that have been preassigned to such a molecule, *i.e.* it enhances ligand-induced gene activity (up to 10-fold) without altering basal transcription levels, and it can reverse interreceptor squelching when transfected into a cell with two active receptors (90). SRC-1 appears to exist in two isoforms and its mRNA is present in all cells. It specifically interacts with the C-terminal activation domain (AF-2) of receptors in a ligand-dependent manner but functions with all steroid/thyroid/retinoic acid receptors tested to date. This coactivator is inactive with receptors bound to pure antagonists but has been shown recently to enhance mixed agonist/antagonist activation of ER as well as ligand-independent activation of receptor by dopaminergic agonists and growth factors. Other potential adaptor proteins that interact with steroid receptors in a ligand-regulated manner, termed receptor-associated proteins (RAPS) or receptor-interacting proteins (RIPS), have been identified, although none have been proven yet to function as transcriptional coactivators. Cells with an abundance of coactivator should have a more pronounced response to a limiting concentration of receptor. It is clear that receptor-coactivator interactions are an important part of the tripartite response system at the gene level and can play a major role in quantitative aspects of cell response.

Elucidation of the molecular interactions of SRC-1 and other coactivators with receptor should advance our understanding of the mechanism of antagonist action. Previous experimental evidence has indicated that agonist- and antagonist-bound receptors exist in distinct conformations (91, 92). Interestingly, agonist-bound receptor binds efficiently to coactivator *in vitro* and *in vivo*, but the antagonist-bound receptor does not bind coactivator. Such differential interactions are illustrated by the varying activities postulated for the different ligand receptor complexes with effector system 2 (Fig. 2, *bottom*) and suggest that antagonist action has its origin at two levels, that of ligand-induced receptor conformation and that of receptor-effector interaction at the genetic level (see below). In such a scheme, antagonist-bound receptor occupies available hormone response elements in the cell, but its conformation does not allow productive interactions with coactivators or the general transcription factor apparatus at the core promoter (TATA box).

Recent data suggesting the existence of a corepressor(s) for the thyroid hormone receptor (and retinoic acid receptor) add an additional twist (93, 94). Unoccupied nuclear thyroid hormone receptor has been reported to silence target gene activity (95, 96). Presentation of thyroid hormone (T_3) reverses silencing and produces a stimulation of transcription. It has been proposed, using reverse squelching experiments to relieve silencing, that a soluble corepressor in target cells binds to unoccupied but not ligand-bound receptor, thus aiding in the thyroid hormone receptor-induced repression of basal transcription at select target genes (93). Recently, two "corepressor" molecules appear to have been cloned in their entirety and seem to fulfill the expected criteria, *i.e.* selective silencing, which is dependent on unoccupied thyroid hormone receptor or retinoic acid receptor (97, 98). In fact, it is likely that multiple coactivators and corepressors will be shown to be operative in cells. More than one agonist-dependent receptor interactive protein has

been reported already (99–105). Although the full consequences of such interactions are not clear at present, an ever increasing level of complexity is evolving at the effector stage of hormone response.

Perhaps the most influential aspect of promoter context or gene-specific response to a ligand is the array of other transcription factors present at a given gene. Although there is evidence for certain promoter-specific factors, the bulk of interactive regulation appears to occur upstream of the transcription start site at multiple enhancers. It is well known that two receptor dimers bound to the 5′-flanking sequence of a target gene can result in transcriptional synergy (106). This also applies to mixes of receptors and other compatible DNA-bound transcription factors, since a number of synergistic (and antagonistic) interactions have been reported among steroid receptors and unrelated transcription factors (72, 73, 73a, 107). Not surprisingly, the mix of receptors with certain transacting factors located at critical positions upstream of the promoter also may result in transcriptional interference.

A number of laboratories have suggested that interactive regulation between transcription factors can occur in cells even in the absence of DNA binding. For example, transcription factor AP-1 can promote active (or positive) influences on receptors independent of their DNA binding. Interactions in the nucleoplasm may occur or AP-1 (fos/jun) may bind to its regulatory element at a gene and serve as a docking site for a steroid receptor via protein-protein interaction (108) (*cf.* Fig. 2, effector system 3). Likewise, in some target genes with unusual estrogen-inducible enhancers, such as c-*myc* (109), creatine kinase (110), cathepsin D (111), and the protooncogene c-*jun* (112), receptor association with other known (such as transcription factor Sp1) or as yet unidentified DNA-binding proteins appears to facilitate receptor interaction with the enhancer. Receptor-mediated gene repression also may occur via protein-protein interactions among transcription factors. For example, glucocorticoid receptor down-regulation of certain genes regulated by the transcription factors AP-1 or NFκB may occur via interactions between such regulators and the glucocorticoid receptor in the absence of DNA binding (113). Finally, nuclear proteins may interact directly and specifically with receptor molecules to antagonize their binding to DNA. Examples of such proteins are calreticulin, which antagonizes steroid receptors (114), and thyroid hormone receptor uncoupling protein (TRUP), which antagonizes thyroid hormone receptor and retinoic acid receptor (115).

Finally, it is worth noting that chromatin structure of genes in their native context provides a significant barrier for receptor to overcome in transcriptional regulation (40, 72, 116, 117). Nucleosomal repression of gene activation must be reversed by receptors, and selected nuclear helper proteins (*e.g.* SWI, SWE, SNF, Sin, etc.) may play important roles in the chromatin remodeling that appears to coincide with induction of transcription. In any event, it is certain that a diverse spectrum of interactions can occur at an effector site and that this complexity may represent a mechanism whereby promoter context and cell specificity of response can be generated.

Pharmacology in Tripartite (Ligand-Receptor-Effector) Systems

In Fig. 2, we have laid out three tripartite schemes to illustrate the types of molecular interactions that may be operating in the activation of gene transcription by nuclear hormone receptors. Through this figure, we also have attempted to represent the combinatorial complexity that can arise as a result of the second interaction, between the ligand receptor complex and the effector. The interactions at the *top* of Fig. 2 illustrate the optimal interaction that might occur between three distinct effector systems and three different ligand receptor complexes, each formed from the same receptor with three different types of ligands; shown is the fact that each ligand-receptor complex has a distinct conformation. At the *bottom* of Fig. 2, we attempt to show the consequence—in terms of signal transduction—of the distinct interaction that each of these ligand-receptor complexes might have with all three of the effector systems. While this illustration is obviously limited and simplified (see previous section "The Nature of Effectors for Nuclear Receptors" and see below), it is meant to capture the conceptual basis of pharmacology in a tripartite receptor system, especially the fact that response diversity can be generated at the level of the effector. In addition to the three scenarios shown in Fig. 2, diversity can also be generated further by differences in the nature of the hormone response element, the influence of neighboring DNA-binding sites for other nuclear factors, as well as the nature of the promoter and chromatin state/conformation.

The transcription activation functions ascribed to different regions of nuclear hormone receptors (AF-1 and 2, or τ1-τ4) can be thought of as sites through which the receptor has the potential for interaction with these various effectors (70, 72, 73). However, the degree to which a particular ligand may engender the receptor to operate through these different activation function sites depends on the nature of the specific effector system with which the ligand-receptor complex interacts. Again, this is dependent on the cell- and promoter-specific factors and the response elements that constitute the effector. In cotransfection systems, mutant receptors can be used to amplify the varied effects of different ligands in their interaction with specific effector sites (5, 41, 43, 118–123). This approach has assisted in the identification of ligands with specific desired biocharacter, such as ligands for the estrogen receptor that have the proper spectrum of agonist/antagonist activity needed for hormone replacement therapy (43).

In tripartite receptor pharmacology, it is useful to consider that the *potency* of a particular ligand is determined principally through the first interaction (*ligand and receptor binding*), whereas its *biocharacter* (*i.e.* agonist-antagonist balance) is determined principally through the second interaction (*receptor-effector coupling*). This may prove to be an oversimplification, as there are known exceptions. In model transcription systems in yeast, certain receptor-modulatory proteins (SSN6-TUP1) have been shown to alter ligand potency (EC_{50}) of both estrogens and progestins by several orders of magnitude, not by a perturbation of ligand receptor binding, but by alteration of receptor-effector coupling that is interpreted as a modification of AF-1 activity. In this system, these adaptor proteins also alter the biocharacter of antiestrogens without changing ligand affinity (89). Related studies have defined a glucocorticoid modulatory element in the tyrosine amino transferase gene, and associated transactivating factors, that alter ligand potency and biocharacter (123). Conversely, it is possible that variations in response element sequence that affect receptor-effector coupling might also alter the conformation of the receptor in a manner that would change ligand affinity. Further investigation of ligand-receptor binding and receptor-effector coupling in carefully controlled systems will be required to fully elucidate the relative role that each interaction plays in establishing pharmacological potency and biocharacter. Regardless of these details, however, *in a tripartite receptor system, the pharmacological parameters of potency and biocharacter are not unique characteristics of a ligand; they can be assigned to a ligand only when reference is made to a specific response or its associated effector* (Table 2).

CONSEQUENCES AND EXPECTATIONS

A prerequisite for receptor pharmacology, be it bipartite or tripartite, is that ligand binding effects some conformational change in the receptor that initiates the response (directly—bipartite) or the potential for response (through coupling with effectors—tripartite). It is clear that the binding of a hormone ligand by its nuclear receptor results in significant conformational changes in the receptor. This has been evident for some time through indirect studies that have noted alterations in thermal stability, antibody binding, heat shock protein dissociation, hydrophobicity, DNA binding, and protease sensitivity upon ligand binding. More recently, crystallographic evidence (124–126) has shown that the small nuclear receptor ligands are almost completely surrounded by protein in their complexes with receptor. Moreover, within this complex there appear to be intimate and detailed contacts between protein and ligand over the whole ligand surface so that, of necessity, the conformation of a steroid-nuclear receptor complex must reflect the shape and structure of its ligand. Thus, the affinity and efficacy with which these conformationally diverse ligand-receptor complexes interact with the various effector sites involved in the transcriptional regulation of different genes reflect the structure of the receptor complex *in its specific ligand-induced conformation.* What are the implications of this ligand-determined conformation of the nuclear hormone receptors?

First, it is not surprising that in the nuclear hormone receptor system, ligands of different structure operating through the same receptor can show distinct cell- and gene-specific effects. One should expect that the same receptor, bound with ligands of different structure, gives rise to complexes of different conformation. Such conformationally different ligand-receptor complexes have the potential for different coupling with the spectrum of effector sites that are present in each target cell and that embody all the cell- and gene-specific factors that enable individual genes to be differentially regulated by different ligands. At the moment, the number of genes whose expression is known to be regulated as a primary response to steroid hormones is rather limited. As more are identified, it is likely that the diversity of response to ligands that is possible with this tripartite receptor system will become even more evident.

Second, in contrast to allosteric effector ligands in enzyme systems and ion channels that bind rapidly to preformed regulatory sites and act like switches controlling the conformation between two states, active and inactive (conformation selection) (127), one should expect the hormonal ligand to affect the conformation of the receptor in more of a progressive or continuum fashion. The rate at which ligands associate with nuclear receptors is slow, far below diffusion control, which characterizes most small molecule-protein interactions. This suggested that the receptor undergoes a substantial conformational reorganization upon binding the ligand. Furthermore, since many unliganded receptors are associated with certain heat shock proteins, the sequences that constitute the ligand-binding pocket were thought to be somewhat disordered in the absence of ligand. Both of these expectations have been confirmed by recent X-ray crystal structures (124–126). Thus, the formation of the ligand-receptor complex in the nuclear hormone receptor system is an excellent example of an induced fit (128), conformation induction (127), or macromolecular perturbation (129), with the receptor conforming to the shape of the ligand (and the ligand, if flexible, having its conformation altered by binding to the receptor as well) (125, 126).

Finally, while structural elucidation methods will soon give us high resolution models for many nuclear receptors binding ligands of varying structure, the impact of this structural information on our understanding of steroid hormone molecular pharmacology, though very useful, will still be limited. The picture will be complete only when the details governing the coupling of these ligand-receptor complexes with the

varying elements of their third partners, the effector sites, also become illuminated.

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Nuclear hormone receptors: ligand-activated regulators of transcription and diverse cell responses

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Signal transduction via nuclear hormone receptors is unusual in that the hormone ligand forms an integral part of the protein complex involved in DNA binding and transcriptional activation. New structural and biochemical results have begun to unravel how these receptors produce different effects in different cells, and the structural changes involved in transcriptional activation.

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Introduction

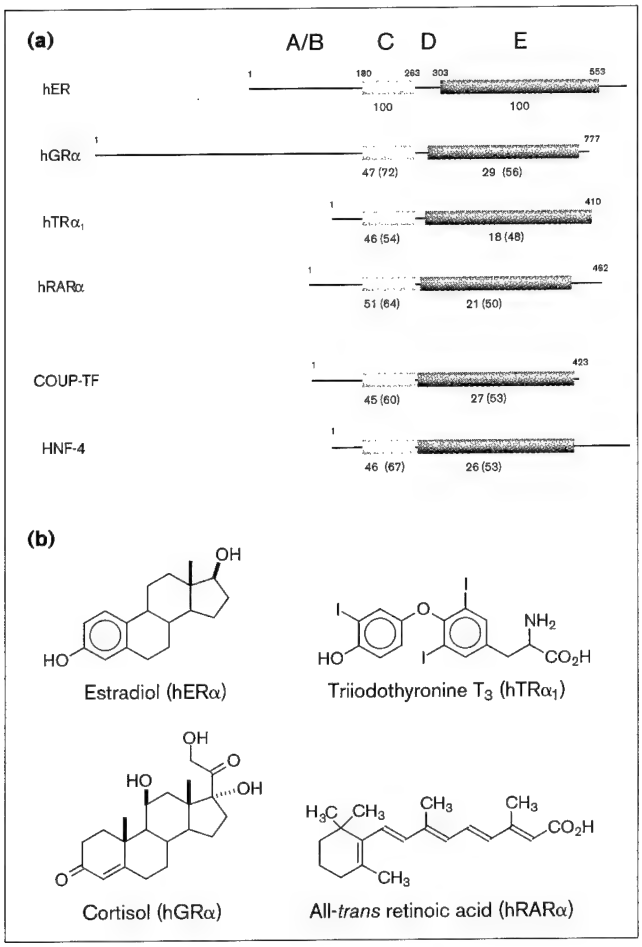
Radiolabeled steroid and thyroid hormones of high specific activity were first prepared in the late 1960's, and were used as probes to identify the sites of hormone action [1]. It has been known for nearly 30 years that these hormones act via intracellular receptor proteins whose principal target for action is in the nucleus. The receptor proteins were quickly surmised to be regulators of transcription [2–6], and are now known to be part of the nuclear receptor superfamily. This large group of transcription factors includes proteins that mediate the action of the steroid hormones (such as estrogens, androgens, glucocorticoids, mineralocorticoids and the insect steroid hormone ecdysone), as well as the non-steroid hormones (for example, thyroid hormone, vitamin D3 and the retinoids) and receptors that mediate the peroxisomal proliferation response to fatty acids and other factors (Fig. 1) [7–11].

Many other members of the superfamily have been identified by low stringency hybridization analysis; some of the genes thus identified encode proteins that are known to be expressed and have the conserved six-domain structure seen in the hormone receptors. Because the hormonal ligands for these proteins are unknown, they are termed 'orphan' receptors [12]. It is however possible that some of these so-called receptors may act as transcription factors alone, without ligands. To add to the complexity of the situation, most classes of receptors within this family contain more than one subtype (i.e., products of closely related genes); sometimes there are also different isoforms (i.e., products from alternate transcription start sites on the same gene) and products of mRNA splice variants. Both the concentration of these receptors and the relative ratio of subtypes and isoforms vary in different target tissues and at different stages of development.

Structure and function of the nuclear receptors

The signature of the nuclear receptor family is a six-domain structure, the most highly conserved portion of which is the small (~70–80 amino acids) domain, C, that is responsible for DNA binding (Fig. 2). This domain has been known for some time to have a helix-loop-helix structure containing two zinc atoms, each chelated by four cysteine thiols at the start of each helix. Three residues at the start of the first helix in this domain 'read' a five to six base pair code in a DNA hormone-response element; the mechanism of this sequence-specific recognition is becoming increasingly clear through structural analysis of domain C–oligonucleotide complexes by X-ray crystallography [13]. The large (~250 amino acid) domain, E, which

Figure 1



Structures of nuclear receptors and their ligands. **(a)** Common domain structure of representative members of the nuclear receptor superfamily, human estrogen receptor α (hERα), human glucocorticoid receptor α (hGRα), human thyroid hormone receptor ($hTR\alpha_1$), human retinoic acid receptor γ (hRARγ), and two orphan receptors COUP-TF and HNF-4. The DNA-binding domain C and ligand-binding domain E are shown with their percent sequence identity (or similarity, in parentheses) to hERα. **(b)** The natural ligands for the first four receptors in (a) are shown; there are no known ligands for the orphan receptors COUP-TF and HNF-4.

is moderately conserved across members of the family, is responsible for hormone binding and dimerization, and is critical in the regulation of transcription (see below). The other domains (the amino-terminal A/B domains, the hinge domain D, and sometimes a carboxy-terminal domain, F), which are poorly conserved in length and sequence across the family, are mostly involved in the modulation of receptor function.

Nuclear receptor ligands are directly involved in transcriptional regulation

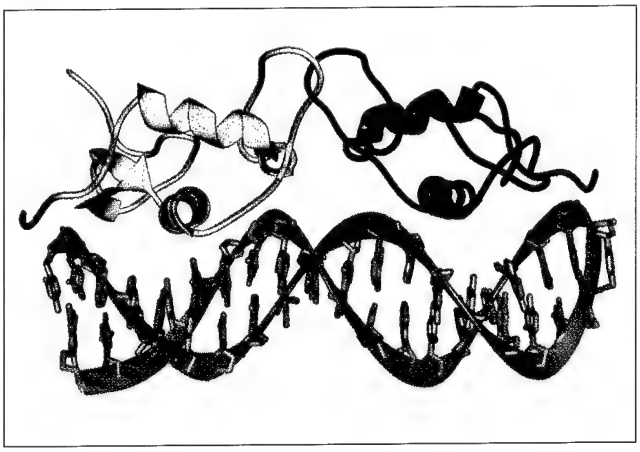
Recent advances have clarified the various ways in which these nuclear receptors can become activated, as well as some of the molecular details of the modulation of the transcriptional activity of specific genes. The essential and intricate role of the ligand in controlling the regulation of gene transcription by these receptors is also now becoming clearer (Fig. 3) [14,15]. Although hormones and growth factors that interact with receptors at the cell membrane may ultimately affect gene transcription, they require multiple-step signal transduction pathways to do so (Fig. 3a); the change in transcription factor activity takes place far away from the interaction between the receptor and the provoking hormonal agent. By contrast, a ligand that activates a nuclear receptor forms a part of the multicomponent complex that directly regulates gene transcription. Such direct interactions offer interesting opportunities for selective pharmacology [16].

There is evidence that high affinity binders for steroid hormones exist in cell membranes, especially in some brain, pituitary and cancer cells. These receptors appear to mediate some very rapid effects of steroid hormones, and it is not yet clear whether their modes of action are similar to or different from the nuclear receptors [17,18]. We will focus here exclusively on the nuclear receptors, since the information on this class is most complete.

Variations on a theme

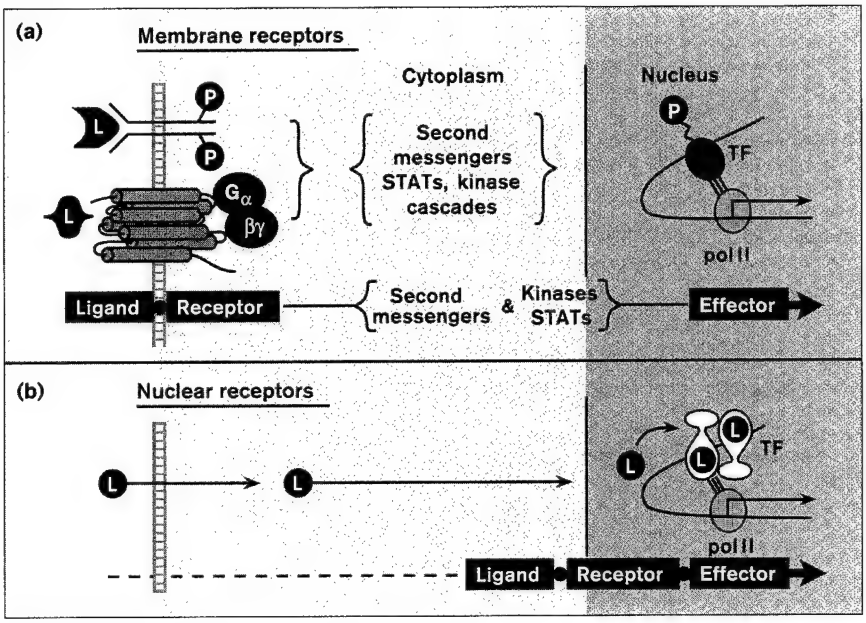
The classical picture of gene activation via nuclear receptors (Fig. 3b) is straightforward. The hormonal ligand binds to the receptor; the receptor–ligand complex thus formed binds (usually as a dimer) to a hormone-response element in the promoter region of a regulated gene, and the transcription of the gene connected to the promoter is thus activated.

Figure 2



A ribbon structure representation of the human glucocorticoid receptor DNA-binding domain dimer complexed with a glucocorticoid response element (GRE). The DNA contact helices, shown edge on, interact with the palandromic DNA sequences of the GRE in adjacent major grooves.

Figure 3

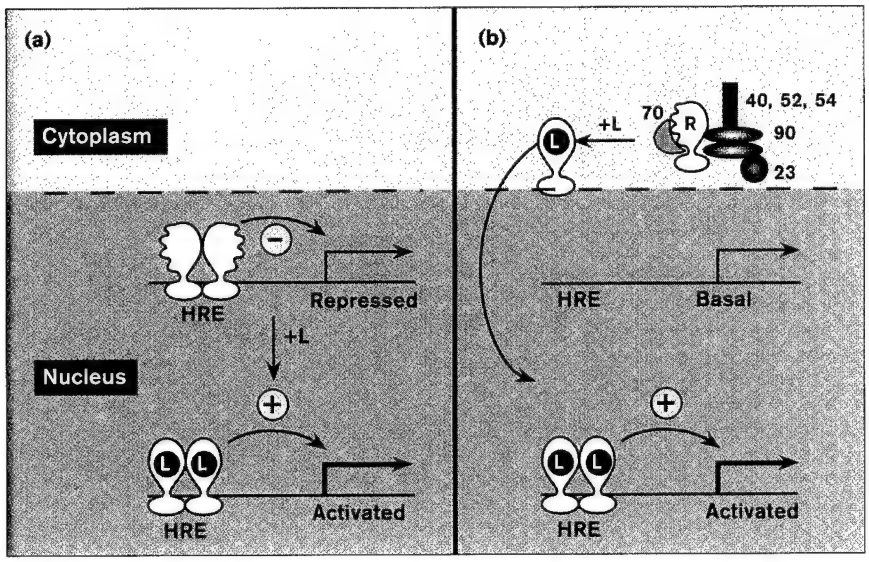


Both membrane receptors and nuclear receptors modulate gene transcription, but nuclear receptors do so more directly. **(a)** Membrane receptor signaling; **(b)** nuclear receptor signaling. In a membrane receptor signaling system, the signal resulting from the binding of the ligand (L) to the receptor must be transduced to the nucleus via complex signal-transduction cascades, which typically involve second messengers, kinase cascades and/or phosphorylation (P) of intermediary proteins (such as STATs) in the cytoplasm. The end result is a change in the activity of a transcription factor (TF) in the nucleus, affecting the rate of initiation of RNA polymerase II (pol II). The effects of a hormone that acts via a nuclear receptor are much more direct; the ligand and receptor form part of the multicomponent complex that modulates pol II activity.

It cannot, however, be this simple. The target of the ligand–receptor complex can clearly vary with cell type, which would be impossible in the rudimentary scheme described above. For example, when estrogen binds to the estrogen receptor in breast cancer and uterine cells the result is the stimulation of transcription from some early response genes, such as *c-myc*, and genes for growth factors (such as TGF-α or pS2) or growth factor receptors (such as the EGF receptor) that are involved in the stimulation of cell proliferation evoked by the hormone [19]. The same ligand-binding event in pituitary and liver cells results in activation of other genes. In the pituitary, the expression of various secreted proteins such as prolactin is increased, whereas in the liver the level of vitellogenin, among others, is increased.

The variations on the classical picture occur at all levels. One source of variability in the effect of ligand binding is the cellular distribution of the receptor in the absence of ligand. The receptors for certain non-steroid ligands (e.g.,

Figure 4



The subcellular location of unliganded nuclear receptors affects the way that they modulate transcription. **(a)** The unliganded receptors for nonsteroid ligands such as thyroid hormone and retinoic acid are typically bound as dimers to their hormone response elements (HREs), even in the absence of ligand, and can act as transcriptional repressors without ligands or transcriptional activators with ligand. **(b)** The unliganded receptors for some steroid hormones, such as glucocorticoids, are largely held as monomers in the cytoplasm by heat-shock proteins (90, 23), chaperonins (70) and immunophilins (40, 52, 54); in this state they have no effect on transcription. Ligand binding releases the receptors from the cytoplasmic aggregate, and the activated receptors bind as dimers to the HREs and activate transcription.

thyroid hormone and the retinoids) appear to be already bound to their response elements (Fig. 4) [20]. Ligand binding may strengthen DNA binding, and may alter the structure of the receptor so as to enhance transcription (see below). In the absence of ligand, these DNA-bound receptors repress gene transcriptional activity [21,22]. In contrast, many of the steroid nuclear receptors (e.g., the glucocorticoid receptor) are largely cytoplasmic in the absence of ligand. They are held in the cytoplasm in complex with heat-shock proteins, chaperonins, and various other proteins such as immunophilins [23]. Ligand binding helps the receptor to shed these proteins, move into the nucleus, dimerize, and interact with appropriate hormone response elements (Fig. 4). In such a scheme, the unliganded receptor cannot be used as a transcriptional repressor, as it is held in the cytoplasm, away from the DNA. The degree of nuclear versus cytoplasmic localization of unliganded receptors varies with different receptors and in different cells, so the effect of the unliganded receptor on transcription will depend on the cell and response in question.

A second level of variation in our originally simple scheme is the way in which the receptor forms a dimer. The nonsteroid nuclear receptors for thyroid hormone, vitamin D and retinoic acid can either form homodimers or heterodimerize with the retinoid X receptor [12,20]. The receptor for the insect steroid hormone ecdysone, on the other hand, is active only as a heterodimer with the protein ultraspiracle, a homolog of the retinoid X receptor (RXR). The preference of the thyroid, vitamin D and retinoic acid receptors for pairing with themselves or with another partner depends on several factors, including the relative concentration of the monomer components (not forgetting the different subtypes and isoforms) and of their cognate ligands. Ligand binding can, in some situations, modulate the formation of specific complexes [24]. A further factor is the structure of the DNA response elements with which the homo- or heterodimers interact [20,24].

The dimerization of steroid receptors at first appeared to be less complicated, since heterodimerization between receptors that bind different ligands (like the thyroid receptor and the RXR) does not seem to occur. Nevertheless, heterodimerization is clearly possible between receptor subtypes (which may have some differences in ligand-binding specificity) and between receptor isoforms (which often have distinctly different transcriptional activities). Examples of subtypes and isoforms that heterodimerize are glucocorticoid receptor α and β, and progesterone receptor A and B forms, respectively. Receptor dimerization and receptor stability are important points for pharmaceutical regulation of transcription via nuclear receptors, and several hormone antagonists (some antiestrogens and antiprogestins, for example) appear to act at this level [25–28].

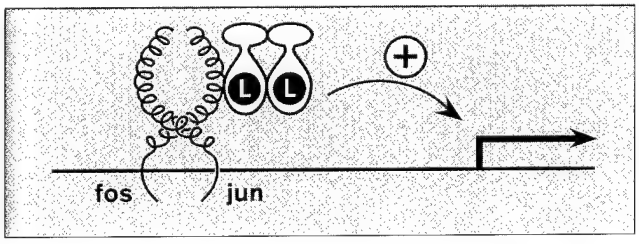
Variations also occur at a third level, the interaction of nuclear receptors with the DNA response elements. Although the response elements are often portrayed as consensus sequences — inverted or direct repeats of a defined five- to six-nucleotide sequence, with various spacers between the repeats — the response elements found in responsive genes are often nonconsensus in sequence; some are half-sites and others have multiple repeats. Often the response elements are found in complex, upstream-enhancer regions, clustered together or even overlapping with response elements for other known transcription factors, which may synergize or compete with the nuclear receptors. Sequences that flank the core response elements can also affect the DNA binding of these receptors (see, for example, [29]). And the structure of the DNA response element, since it affects the recognition between the receptor and the DNA, may also affect the interaction between the receptor and the ligand.

Given all the sources of variation described above, especially the fact that nuclear receptors may interact with or compete with a number of other sequence-specific transcription factors, it is not surprising that the response to a specific hormone depends on both the cell in which it is acting and the gene whose activity it modulates [16].

Nuclear receptor activation without direct DNA binding or without ligand binding

A curious but major deviation from the classical scheme for nuclear receptor action is gene activation in the absence of direct DNA-binding by the receptor. In this situation the promoter for a gene whose activity is clearly regulated by a nuclear receptor and its hormone appears to have no hormone-response element for the receptor, and does not, in fact, require direct DNA binding by the receptor. The hormone–receptor complex seems to function by binding to DNA indirectly via other DNA-tethered transcription factors (see, for example, [30–32]), thus acting as a ligand-modulated co-regulator, rather than a ligand-modulated transcription factor (Fig. 5).

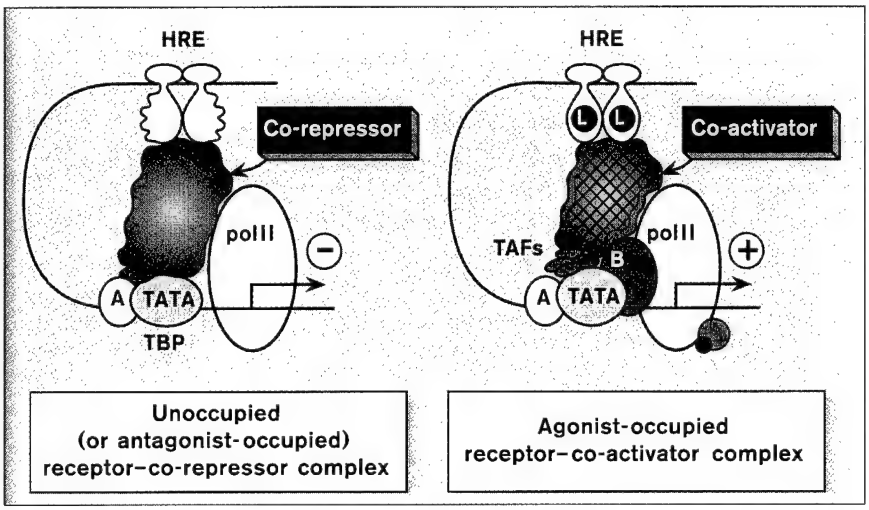
Figure 5



Nuclear receptor gene activation can occur without direct DNA binding. The nuclear receptor is tethered to DNA by a protein–protein interaction with another sequence-specific transcription factor, such as fos/jun (AP1). In such a case, the nuclear receptor has the role of a ligand-modulated co-activator of transcription.

Figure 6

Co-regulators mediate the interaction between the nuclear receptor and components of the transcription complex. Unoccupied or antagonist-occupied receptors can recruit co-repressors (left); when an agonist ligand binds, the ligand–receptor complex can recruit co-activators (right).



Another major deviation from the classical scheme for activation of genes by nuclear receptors is ligand-independent gene activation. In certain systems there appears to be significant crosstalk between signal-transduction pathways that activate transcription. The result is that growth factors or hormones that operate through receptor tyrosine kinases or via cAMP or other second messengers can activate nuclear receptor regulated genes in a manner that requires receptor but not ligand [19]. In some cases, these alternative pathways may synergize with the normal ligand-mediated pathway [33]. The molecular mechanism for such action is not well understood, but it is possible that phosphorylation of specific sites on the nuclear receptors may enhance the transcriptional activity of the unliganded receptor [19,34].

Modulation of gene transcription

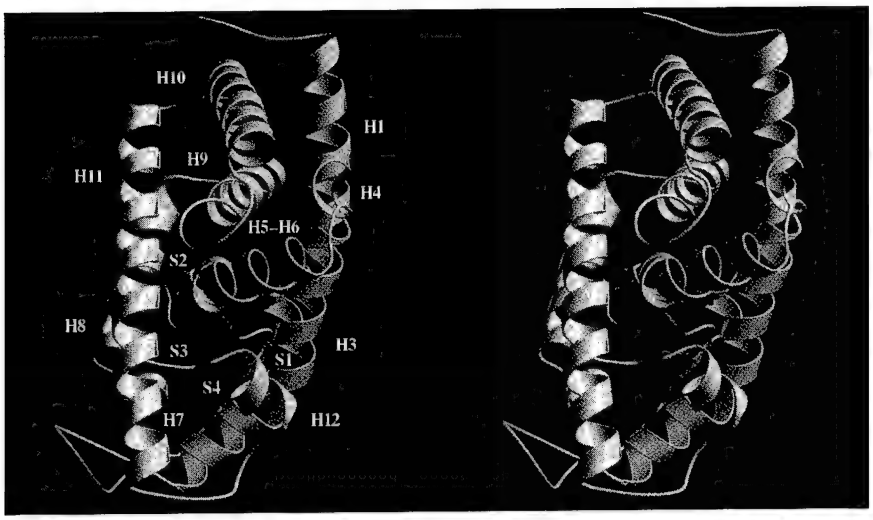
Once a nuclear receptor is bound to DNA, what happens next? The final step of the classical pathway, the process by which these receptors modulate the rate of gene transcription (Fig. 6), has its own sources of regulatory complexity. First, it is important to recognize that the rate at which a gene is transcribed depends both on the local chromatin architecture, and on the rate at which an active RNA polymerase preinitiation complex can be assembled. The nuclear receptors appear to affect both of these processes, both directly and indirectly via 'transcription intermediary factors' (TIFs) [9,35,36], although their effect on chromatin architecture is poorly understood. There is evidence that DNA-bound nuclear receptors interact directly with some of the proteins comprising the basal transcription machinery, such as TFIIB or TATA-binding protein associated factors (TAFs) [37–39]. If they suppress or stimulate a rate-limiting step in the assembly of an active RNA polymerase II preinitiation complex, this would result in repression or activation of transcription. In many cases the relevant interactions between nuclear receptors and basal transcription factors appear not to be direct, however, but are mediated by various co-regulators.

The co-regulators involved in nuclear-receptor modulation of gene transcription are diverse, and are being discovered at an increasing rate. They are often large multidomain proteins, with some homology to factors that are known to modulate chromatin structure; some have known protein-interaction domains, or have the ability to interact with various components in the general transcription apparatus [16]. Some also appear to fit nicely into the unliganded-repression/liganded-activation paradigm, in that one set of co-regulators binds to the unliganded thyroid and retinoid receptors to repress transcription [40,41], whereas another set binds to liganded receptor to enhance transcription [42–46]. In the case of the steroid receptors, the co-regulators appear to bind to either the amino-terminal or carboxy-terminal activation domain of the receptors. Some co-regulators interact with and influence the transcriptional activity of many steroid hormone receptors and other related receptors, such as RXR, whereas other co-regulators show a more restricted range of receptor interaction.

Structural and conformational changes on ligand binding

As the interaction between the co-regulators and the nuclear receptor is regulated by ligand binding, it is plausible that ligand binding elicits a conformational change in the receptor that may permit co-activator but not co-repressor binding in the presence of ligand (or co-repressor but not co-activator binding in the absence of ligand). Mutational mapping studies have begun to identify the different regions of the receptor that seem to be responsible for interaction with co-repressors and co-activators [35,43,47]. Most exciting are some of the structural features revealed

Figure 7



Stereoview of a ribbon structure of the ligand-binding domain of the rat thyroid hormone receptor complexed with thyroid hormone (T3), shown as a skeletal structure in the lower half of the protein. The regions of α-helical (H) and β-strand (S) secondary structure are designated.

in the recent X-ray crystal structures of three different receptor ligand-binding domains (domain E) (Fig. 7); these structures provide insight into the conformational reorganization that occurs upon ligand binding [48–51].

The ligand-binding domain of the nuclear receptors is large, larger than most single protein domains, with a unique antiparallel α-helix triple sandwich topology (Fig. 7). Approximately half of the domain consists of a rigid, tightly packed assembly of helices that appear to act as a fundament or fulcrum for the action of the remainder of the domain, which is more flexible and is involved in ligand binding. Although the three structures that have been described so far do not permit a direct comparison between the conformations of a single receptor in the liganded and unliganded state, certain general features have emerged that are likely to hold true for the ligand-induced conformational changes of all of the members of the superfamily.

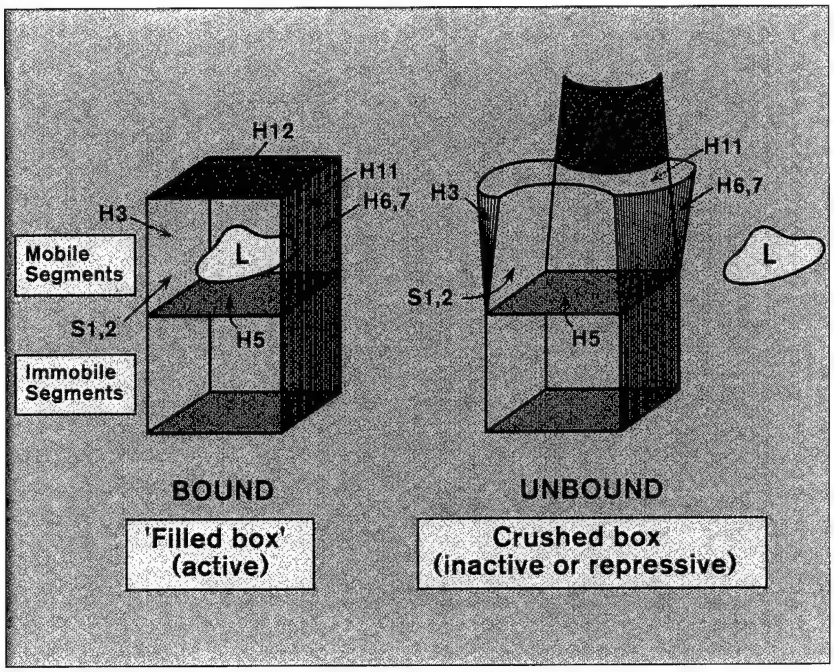
In the bound state, the ligand is completely engulfed by the flexible portion of the domain, and actually forms the hydrophobic core for this region [49,50]. Six segments of secondary structure, arranged roughly as the six sides of a box, surround the ligand, with more than 20 residues making direct contact with the ligand (Fig. 8). In the liganded state, the carboxy-terminal portion of this domain, an amphipathic helix, termed the activation function 2 activation domain (AF2-AD), interacts with the ligand and is positioned adjacent to two other helical portions of the receptor whose specific orientation is also dependent upon contacts with the ligand (see Fig. 7, helix 12). This composite surface, whose integrity appears to be critically dependent on ligand binding, is one likely site for co-activator binding.

By contrast, in the unbound state, the flexible portion of the ligand-binding domain lacks its hydrophobic core, namely, the ligand. In the one published structure for an unliganded receptor [48], the box-like structure of the flexible portion of domain E appears to have collapsed, with two sides tipping inward and two sides tipping outward; the activation helix is dislodged from its position between the other two helices, since their relative position is no longer supported by contacts with the ligand (see Fig. 8). The composite surface for co-activator binding is thus absent or at least substantially modified in the unliganded state. But in the collapsed state, various new topographical features have developed, providing potential sites for co-repressor binding.

X-ray crystallography provides static pictures of protein structure. It is thus possible that the flexible ligand-binding region of domain E in the unliganded state may be rather fluid, perhaps in a molten globule-like state. The binding of heat shock proteins (which normally bind only to unfolded or partially folded proteins) and immunophilins to the unliganded steroid receptors and the sensitivity of the unliganded receptor to proteolysis supports this view [52]. Further studies, especially ones in which a direct comparison can be made between structures of the liganded and unliganded state of the same receptor, will be needed to verify the generality of these conformational transitions.

Ligand binding affects receptor shape — thus, receptor shape reflects ligand shape. As co-repressor/co-activator binding responds to alterations in receptor shape, the ligand is the crucial factor in recruiting or disbanding these important co-regulators. The view that ligand shape determines receptor shape and thus receptor activity can also account for the spectrum of biological activity — from pure agonists to partial agonists/antagonists to pure antagonists — that is known for ligands for some of these nuclear

Figure 8



A 'box model' for the ligand-binding domain of a nuclear receptor. When an agonist ligand is bound, the upper box, made up of mobile segments with the ligand at its core, is 'filled'; in this conformation, it has a structure in which the activation domain (helix 12) is in the active state, where it can interact with co-activators, activating transcription. Without ligand, the upper box is empty and is 'crushed', so that two sides cave inward and two sides bulge outward; the activation helix is displaced from the active state, and the empty receptor is thus either inactive or recruits co-repressors to become repressive. Antagonists and partial agonists fill the top box in a different manner, such that the activation helix is fully or partially misoriented from the activating position. The conformation of the lower box is not affected by ligand binding. (Note that this schematic representation of the ligand binding domain of a nuclear receptor is shown in the orientation opposite to that of the thyroid hormone receptor–T3 complex shown in Figure 7. In Figure 7, the ligand-binding 'box' is at the bottom.)

receptors, such as estrogens and progestins. Given all this, the potential for pharmaceutical modulation of the transcriptional activity of nuclear receptors is obvious [15,16].

Pharmacological issues, however, extend beyond the ligand–receptor interaction. The biological effect that a particular ligand will have, acting via a given receptor, will also depend on the intracellular context (i.e., the levels of the relevant co-regulators and transcription factors with which the receptor cooperates) and the promoter for the specific gene being regulated (i.e., the structure of the hormone-response element and whether any other transcription factors bind to nearby sites). This 'tripartite receptor pharmacology', comprising ligands, receptors, and cell and promoter specific transcriptional effectors, offers rich possibilities for developing tissue- and response-specific pharmaceuticals [16].

The future

There is much more to learn. The details of the ligand-induced conformational changes within one receptor protein are yet to be revealed, and we do not yet know how all the different domains of a nuclear receptor interact with each other. A full appreciation of the molecular interactions involved in the gene-regulating action of the nuclear receptors will require reconstitution of multiprotein complexes involving the intact receptor (as a homo- or heterodimer) interacting with a complete gene regulatory region, together with other associated transcription factors, co-regulator proteins, and elements of the general transcription apparatus. Equally important will be biological studies detailing regulation of the levels and activity of receptors and their co-regulators as a function of physiological and developmental state in different hormonal target cells and tissues. Clearly, the major and perhaps the most exciting challenges still lie ahead.

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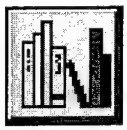

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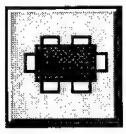

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Response-specific Antiestrogen Resistance in a Newly Characterized MCF-7 Human Breast Cancer Cell Line Resulting from Long-term Exposure To *Trans*-hydroxytamoxifen

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To understand better the antiestrogen-resistant phenotype that frequently develops in breast cancer patients receiving tamoxifen, we cultured MCF-7 breast cancer cells long-term (>1 yr) in the presence of the antiestrogen *trans*-hydroxytamoxifen (TOT) to generate a subline refractory to the growth-suppressive effects of TOT. This subline (designated MCF/TOT) showed growth stimulation, rather than inhibition, with TOT and diminished growth stimulation with estradiol (E_2), yet remained as sensitive as the parental cells to growth suppression by another antiestrogen, ICI 164,384. Estrogen receptor (ER) levels were maintained at 40% of that in parent MCF-7 cells, but MCF/TOT cells failed to show an increase in progesterone receptor content in response to E_2 or TOT treatment. In contrast, the MCF/TOT subline behaved like parental cells in terms of E_2 and TOT regulation of ER and pS2 expression and transactivation of a transiently transfected estrogen-responsive gene construct. DNA sequencing of the hormone binding domain of the ER from both MCF-7 and MCF/TOT cells confirmed the presence of wild-type ER and exon 5 and exon 7 deletion splice variants, but showed no point mutations. Compared to the parental cells, the MCF/TOT subline showed reduced sensitivity to the growth-suppressive effects of retinoic acid and complete resistance to exogenous TGF-β1. The altered growth responsiveness of MCF/TOT cells to TOT and TGF-β1 was partly to fully reversible following TOT withdrawal for 16 weeks. Our findings underscore the fact that antiestrogen resistance is response-specific; that loss of growth suppression by TOT appears to be due to the acquisition of weak growth stimulation; and that resistance to TOT does not mean global resistance to other more pure antiestrogens such as ICI 164,384, implying that these antiestrogens must act by somewhat different mechanisms. The association of reduced retinoic acid responsiveness and insensitivity to exogenous TGF-β with antiestrogen growth resistance in these cells supports the increasing evidence for interrelationships among cell regulatory pathways utilized by these three growth-suppressive agents in breast cancer cells. In addition, our findings indicate that one mechanism of antiestrogen resistance, as seen in MCF/TOT cells, may involve alterations in growth factor and other hormonal pathways that affect the ER response pathway.

J. Steroid Biochem. Molec. Biol., Vol. 58, No. 0, pp. 1–14, 1996

INTRODUCTION

Tamoxifen is the most common endocrine therapy used in the treatment of estrogen receptor-positive breast cancer. Unfortunately, the vast majority of tamoxifen-treated breast tumors eventually become refractory to the beneficial effects of this antiestrogen. Characterization of tamoxifen-resistant breast tumors established in nude mice [1, 2] and in culture [3–6] has shown that reductions in estrogen receptor (ER) content or changes in ER binding affinity for ligands are not necessarily causative factors in antiestrogen re-

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sistance. Whereas changes in ER-mediated transcriptional activity may confer or promote antiestrogen resistance, it is also possible that this phenotype may be influenced by interactions with other regulatory pathways. There is an emerging body of evidence that shows cross-talk of the ER pathway [7, 8] with peptide growth factors and with other nuclear receptor ligands, such as the retinoids [9–11] suggesting that these may be involved in antiestrogen resistance and in the more aggressive behavior often associated with antiestrogen-resistant tumors.

In normal and neoplastic epithelial cells, the transforming growth factor-βs (TGF-βs) are most frequently associated with growth inhibition, whereas in a number of cell types, such as fibroblasts, the TGF-βs are growth stimulatory (for review, see [12]). The finding that tamoxifen increases TGF-β levels in tumors suggests that the therapeutic effect of tamoxifen in slowing or arresting tumor growth may be partly attributable to the growth-inhibitory action of the TGF-βs [13]. It has been demonstrated that estrogens suppress and antiestrogens augment TGF-β expression in human breast cancer cell lines in culture [14, 15]. Interestingly, a number of advanced stage tumors and cancer cell lines exhibit a TGF-β-resistant phenotype (for example, [16]), suggesting that the development of TGF-β resistance may abrogate the beneficial effects of tamoxifen on breast cancer cells.

We maintained MCF-7 human breast cancer cells in *trans*-hydroxytamoxifen (TOT) for more than 1 year to generate an *in vitro* model for the study of tamoxifen resistance. Herein, we report on the proliferation of the cells, and the activity of the estrogen receptor and its responsiveness to estrogen and to two different classes of antiestrogens, as well as on the effects of TGF-β and retinoic acid on this subline. Our findings suggest interrelationships among the pathways utilized by antiestrogens, TGF-β and retinoic acid in the regulation of these breast cancer cells.

MATERIALS AND METHODS

Materials

Radioinert E_2 and R5020 (promegestone; 17,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione), nutritional supplements for growth in serum-free conditions, protease inhibitors, TPA (12-O-tetradecanoylphorbol-13-acetate), MTT (thiazolyl blue), all-*trans*-retinoic acid and sera were purchased from Sigma Chemical Co. (St Louis, MO). *Trans*-hydroxytamoxifen (TOT), ICI 182,780 and ICI 164,384 (ICI) were generously provided by Zeneca Pharmaceuticals (Macclesfield, U.K.). Tissue culture media and antibiotics were purchased from GIBCO (Grand Island, NY). Tritiated E_2 (2,4,6,7-^{3}H-N-estradiol) and ^{3}H-R5020 (17-alpha-methyl-^{3}H-promegestone) were purchased from New England Nuclear Corp. (Boston, MA) and methyl[^{3}H]thymidine from ICN, Costa Mesa, CA.

Cell culture

MCF-7 human breast cancer cells were acquired from the Michigan Cancer Foundation; cells between passage numbers 150 and 300 were used in these studies. Parent MCF-7 cells were routinely cultured in phenol red-containing Eagle's minimal essential medium (MEM) supplemented with 5% heat-inactivated fetal calf serum (FCS), E_2 (10^{-12} M), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (10 mM), insulin (6 ng/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml). To generate TOT-resistant MCF-7 sublines, cells were maintained in the above media without supplemented E_2, and with 10-fold increases in TOT concentration (10^{-9} M–10^{-6} M) every four weeks. The cells were thereafter routinely maintained with 10^{-6} M TOT. Cells were subcultured weekly at near confluence using 1 mM EDTA prepared in Hank's balanced salt solution and medium was replenished every other day. To generate clonal-derived sublines, 96-well plates were seeded at approximately one cell every three wells. Two weeks after seeding, wells containing only one colony were identified. Clonal-derived sublines were maintained and sequentially transferred to 24-well plates, then six-well plates and T25 flasks.

For all studies involving experimental treatments, cells were grown without E_2 for one week or without TOT for two weeks and then subsequently in 5% CDFCS IMEM without insulin for an additional 5–10 days prior to the experiment, in order to deplete the cells of E_2 or TOT prior to the onset of experiments.

Cell proliferation studies

To determine cell number, cells were seeded at 150,000 cells/T25 flask in triplicate and after two days day 0 flasks were counted and the medium was replaced and treatments added. Media were changed every two days and cells in logarithmic phase were harvested on day six and counted in a Coulter particle counter (Hialeah, FL).

Anchorage-independent growth was determined by a colony-forming assay. In brief, six-well plates were coated with 0.6% agar in 5% CDFCS IMEM and allowed to cool. Cell suspensions containing 10,000 cells were passed through a 22-gauge needle and then added to a mixture equilibrated to 45°C containing 0.4% agar, 5% CDFCS IMEM and treatments and added to the wells. Plates were grown for two weeks with a top layer of media which was replenished every three days. Colony size (>60 μ) was determined microscopically with an ocular grid (Wild M40 microscope; Heerbrugg, Switzerland).

In some studies, cell number was determined by the MTT assay. MTT (thiazolyl blue) is converted from a yellow-colored salt to a purple-colored formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenases, the activity of which is linear with cell number. Cells were seeded at 2000–5000 cells/well in 96-well plates in quadruplicate. After treatment as indicated, 50 μl of 2 mg/ml MTT was added and plates were incubated at 37°C for 4 h. Wells were drained and formazan crystals were solubilized in 150 μl buffer (20% w/v sodium dodecyl sulfate dissolved in 50% dimethylformamide/50% dH_2O containing 2.5% acetic acid and 2.5% of 1 N HCl with a final pH of 4.7 [17]. Absorbance at 570 nm was determined on a plate reader.

For [^{3}H]thymidine incorporation studies, cells were seeded at 2000 cells/well in 24-well dishes. Two or three days later the wells were washed in serum-free media for 2 h and then treated in serum-supplemented or in serum-free IMEM with 1 μg/ml fibronectin, 2 μg/ml transferrin and 1:100 dilution of trace elements. After three or four days, the cells were incubated with 0.5 μCi methyl[^{3}H]thymidine at 37°C for 2 h. Plates were sequentially washed and fixed with ice-cold PBS, 10% TCA (2×), MeOH, and then incorporated label was recovered by incubation of the wells in 0.5 N NaOH for 30 min at 37°C. Lysates were transferred to vials containing ScintiVerse™ cocktail (Fisher Scientific) and [^{3}H]thymidine was determined in a scintillation counter.

Whole cell binding assays

Whole cell ER and progesterone receptor (PgR) binding assays were done as previously described [18]. Cells were incubated with 10 nM [^{3}H]E_2 or [^{3}H]R5020 in the absence or presence of a 100-fold excess of unlabelled ligand, and for PgR studies, with 3.75 ng/ml hydrocortisone. After incubating at 37°C for 40 min, cells were washed three times with 1% Tween–80 in phosphate-buffered saline and bound radiolabelled ligand was extracted with ethanol and counted in a scintillation counter.

Western blot analysis

Subconfluent cell layers were pelleted and resuspended in 50 mM Tris (pH 7.4), 7.5 mM EDTA, 0.6 M NaCl, 10% glycerol in the presence of proteinase inhibitors (leupeptin, pepstatin A, phenylmethylsulfonylfluoride) and homogenized on ice. Samples were centrifuged for 25 min at 46 K and the protein content in the supernatants determined in a BCA assay (Pierce Chemical Co., Rockford, IL). Samples (150 μg) were boiled for 5 min in loading buffer, separated on a SDS polyacrylamide stacking gel and transferred to nitrocellulose. Blots were incubated with estrogen receptor-specific antibodies H222 (exon 7 epitope) or with H226 (exon 1,2 epitope) in combination with D547 (exon 4 epitope), then a bridging rabbit anti-rat IgG, and finally with [^{125}I]protein A, and then exposed to film [19].

TGF-β protein determinations

Subconfluent cell layers were washed three times for 1 h in serum-free media and then incubated in serum-free media supplemented with 2 μg/ml transferrin, 1 μg/ml fibronectin and 1:100 trace elements. After 48 h, BSA was added to the conditioned media to a final concentration of 0.5 mg/ml and the samples were snap frozen and later tested for the ability to inhibit [^{3}H]thymidine incorporation by MV 1 Lu mink lung epithelial cells. Latent and total TGF-β bioactivity was kindly determined by Anita Roberts and Nan Roche of NCI, Bethesda, MD as described [20].

Transient transfections and assays for reporter activity

To measure responsiveness to E_2, a construct containing the consensus estrogen response element linked to a thymidine kinase promoter and the CAT gene (ERE-tk-CAT) was cotransfected into cells along with the internal control plasmid, CMV-β-gal, exactly as described [20] and cell extracts were assayed for CAT activity. Fold inductions within each assay were normalized against β-galactosidase activity as described [20].

Isolation of RNA

Isolation of total RNA from near confluent cell monolayers was performed using guanidinium thiocyanate-phenol-chloroform extraction with some modifications as described [20].

Northern blot analysis

For studies involving the induction of pS2 mRNA, cells were pretreated in 5% CDFCS IMEM as described in the Materials and methods section and treated with the ligands indicated for 12 h. Twenty micrograms total RNA were separated by electrophoresis, transferred to a nylon support and hybridized with random primer labelled fragments of human pS2 cDNA [21]. Sizes of bands were confirmed by comparison to a 0.24–9.5 kb RNA ladder (GIBCO BRL, Grand Island, NY).

Ribonuclease protection assays

Ten to 30 μg of RNA was co-precipitated with *in vitro* transcribed, gel purified cRNA labelled with phosphorus-32 and resuspended in 80% formamide/0.1 M Na citrate (pH 6.4)/0.3 M NaOAc (pH 6.4)/1 mM EDTA. Samples were heated to 85°C for 5 min and hybridized overnight at 45°C. Unhybridized total RNA and probe was digested in a final concentration of 5 units/ml RNase A and 1000 units/ml RNase T1 for 30 min at 37°C. The sizes of protected fragments were confirmed by comparison to a lane loaded with a 0.16–1.77 kb RNA ladder (GIBCO). The probes

used were a 240 bp Mbo II segment of TGF-β1 cDNA, a Hpa 1 segment of TGF-β2/sp72 cDNA, and a 125 bp Nde 1 segment of TGF-β3 cDNA as described previously [20]. The probes for TGF-β Type I and II receptors were a 300 bp unprotected Hinc II fragment (220 bp protected fragment) and a 360 bp unprotected Xho I fragment (260 bp protected fragment), respectively, kindly provided by Dr M. Brattain. A 125 bp fragment of human β-actin (Ambion Inc., Austin, TX) was used as an internal control. The relative intensity of the bands was quantitated on an UltraScan XL densitometer using GelScan XL evaluation software.

[^{125}I]TGF-β1 binding assay

Cells at 75–90% confluency in 24-well plates were washed three times over 1 h with serum-free media supplemented with 0.1% BSA and incubated with 10^{-10} M [^{125}I]TGF-β1 with or without a 100-fold excess of cold TGF-β1 for 45 min. Cells were then washed four times with 0.1% BSA in ice-cold HBSS and solubilized with 1% Triton X-100/20 mM HEPES, pH 7.4/10% glycerol/0.01% BSA for 15 min at 37°C. Solubilized fractions were counted in a gamma counter [20].

RT–PCR amplification, cloning and sequence analysis

Samples of RNA, isolated from parental MCF-7 and MCF/TOT cells as described above, were reverse transcribed by AMV reverse transcriptase (Promega Corp., Madison, WI) and amplified using sense and antisense primers specific for sequences flanking the hormone binding domain of the estrogen receptor (forward primer corresponding to estrogen receptor cDNA nucleotides 1036–1052, and reverse primer corresponding to nucleotides 1946–1967, respectively) using a PTC-100 programmable thermal controller (MJ Research Inc.). Products were separated and purified from agarose gel electrophoresis and sequenced directly (Sequenase version 2.0; U.S. Biochemical Corp.), according to Newton *et al.* [22]. Sequencing reactions were analysed on 6% denaturing polyacrylamide gels. Sequences were compared to that reported for the human estrogen receptor in the Genetic Sequence Data Bank (EMBL/GenBank).

RESULTS

Growth responsiveness of parent MCF-7 and MCF/TOT cells to estrogen and antiestrogens

To generate TOT-resistant MCF-7 sublines, cells were cultured with 10-fold increases in TOT concentration (10^{-9} M–10^{-6} M) every 4 weeks, as described in Materials and methods. The cells were thereafter routinely maintained with 10^{-6} M TOT in their culture medium. Under this regimen, dramatically slowed growth rates were observed for approximately 30 weeks from initial TOT exposure, after which time cell growth rates progressively increased. The experiments described herein were conducted between 60 and 140 weeks of maintenance on TOT, during which time population doubling rates were compar-

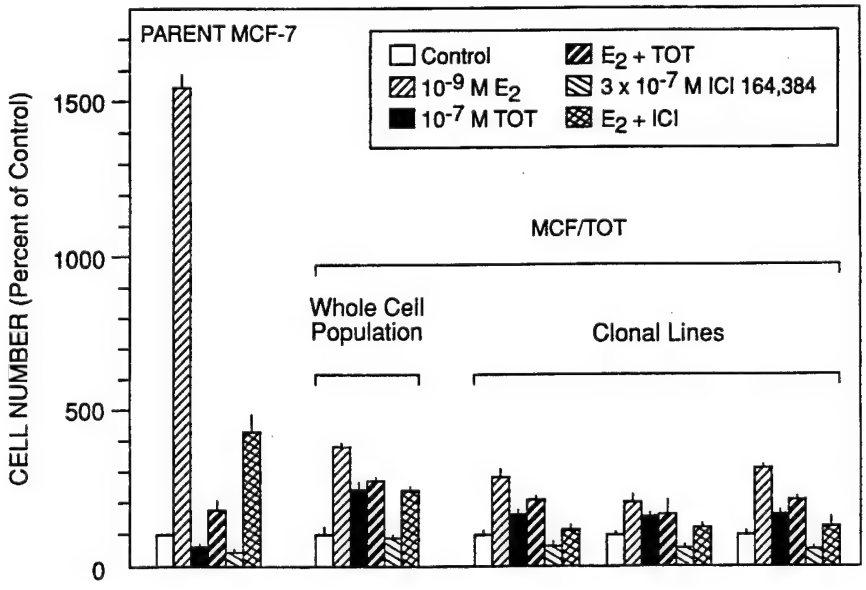

Fig. 1. Anchorage-dependent growth responsiveness of parent MCF-7 and MCF/TOT cells to estrogen and antiestrogens. Cell number in triplicate T25 flasks was determined on day 6 of treatment with the indicated compounds. Treatments were with 10^{-9} M E_2, 10^{-7} M TOT, and 3×10^{-7} M ICI 164,384 alone or together. Values are expressed as percentage of cell number in ethanol-treated control flasks. Cells were depleted of steroids and TOT for 3 weeks prior to the onset of the experiment as described in Materials and methods. The basal growth rates of the MCF-7 and MCF/TOT sublines were 3.95 ± 0.01 and 3.34 ± 0.07 days/population doubling, respectively. Data represent mean $\pm$ SEM ($n = 3$).

able in the parent MCF-7 and MCF/TOT cells (1.3 ± 0.1 and 1.6 ± 0.1 days, respectively). To determine the proliferative effects of estrogen and antiestrogens on parent MCF-7 and MCF/TOT cells, growth rates were slowed to approximately 3–4 depopulation doubling by transfer from steroid- and/or TOT- and phenol red-containing media to media lacking phenol red [23] and TOT and depleted of steroids by charcoal-dextran treatment of the serum. Parent MCF-7 cells exhibited dramatic increases in cell proliferation rate in response to treatment with 10^{-9} M E_2 ($1535 \pm 374\%$ of control; Fig. 1). Treatment with the pure antiestrogen, ICI 164,384 (ICI), partly reversed estrogen-stimulated growth ($432 \pm 163\%$) and was growth suppressive when administered alone ($44 \pm 10\%$). Similar results were found when a structurally related pure antiestrogen, ICI 182,780, was used (data not shown). Treatment with the antiestrogen TOT reduced the growth of the parent MCF-7 cells ($61 \pm 9\%$ of control) and also very effectively suppressed the proliferation of these cells stimulated by E_2.

MCF/TOT cells were growth stimulated by 10^{-9} M E_2 ($387 \pm 54\%$; Fig. 1), but this response was modest compared to the dramatic effect of estrogen stimulation on the parent MCF-7 cells. Interestingly, we found that the effect of treatment with TOT shifted from growth suppression, as observed in the parent MCF-7 cells, to growth stimulation in the MCF/TOT subline ($247 \pm 59\%$). These results suggest that MCF/TOT cells were not refractory to TOT, but instead interpreted this ligand as an agonist. Treatment with the pure antiestrogen, ICI 164,384, reduced the growth of MCF/TOT cells slightly ($68 \pm 17\%$), and partly reversed E_2-stimulated growth ($242 \pm 32\%$), as did ICI 182,780 (data not shown). This indicates that MCF/TOT cells were not cross-resistant to pure antagonists of the estrogen receptor.

We were also interested in determining whether the altered phenotype of the MCF/TOT subline was homogeneous or heterogeneous within the cell population. Clonal lines were found to exhibit a growth phenotype similar to that of the MCF/TOT whole cell population (Fig. 1).

MCF/TOT cells showed responses to estrogen and antiestrogen in anchorage-independent colony formation assays (Fig. 2) similar to those observed in the anchorage-dependent cell proliferation assays of Fig. 1. MCF/TOT cells grown in soft agar were E_2 stimulated in terms of colony formation, although to a lesser extent than the parent MCF-7 cells (Fig. 2), and MCF/TOT cells were also growth stimulated by TOT and growth inhibited by ICI 164,384. In contrast, parental MCF-7 cells were inhibited by both TOT and ICI 164,384. Interestingly, ICI 164,384 reversed the growth stimulation observed in MCF/TOT cells in response to treatment with TOT.

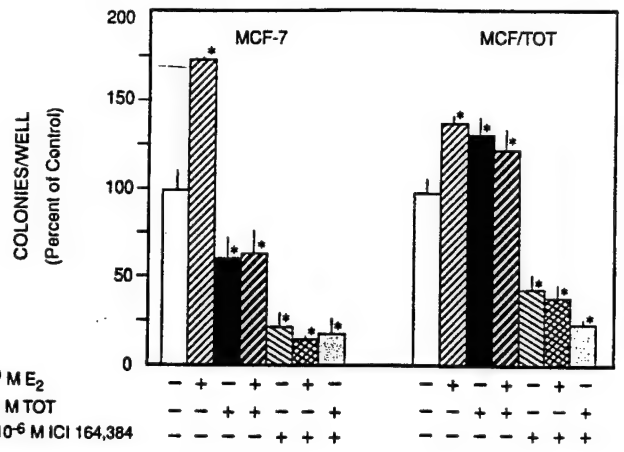

Fig. 2. Anchorage-independent growth responsiveness of parent MCF-7 and MCF/TOT cells to estrogen and antiestrogens. Parent MCF-7 and MCF/TOT cells were seeded at 10,000 cells/well in six-well plates in a top layer of 0.4% agar, 5% CDFCS IMEM and treatments and over a solidified bottom layer of 0.6% agar in 5% CDFCS IMEM. Colonies larger than 60 were counted microscopically with an ocular grid on day 14 of treatment. Colony number from ethanol-treated control wells was not dramatically different between the MCF-7 and MCF/TOT sublines (838 ± 45 and 951 ± 126 colonies/well, respectively), nor from two separate clonal-derived sublines of MCF/TOT cells (1014 ± 430 colonies/well; data not shown). Values are expressed as percentage of colony number $\pm$ SEM of ethanol-treated control wells from three separate experiments; *value significantly different from the control treatment at $P < 0.05$ by Student's t-test.

Assessment of antiestrogen antagonism of estrogen-stimulated growth and pS2 mRNA expression

Treatment with TOT abolished E_2-stimulated growth in parent MCF-7 cells in a dose-dependent manner (Fig. 3, panel A). Fifty per cent suppression was achieved with ca. 1×10^{-9} M TOT, and the highest concentration of TOT tested (2×10–6 M) gave nearly complete suppression of E_2-stimulated growth in parental MCF-7 cells. MCF/TOT cells were much less sensitive to suppression of E_2-stimulated growth by TOT (Fig. 3, panel A). No suppression of E_2-stimulated growth was seen until concentrations of TOT greater than 2×10^{-9} M were used, and 50% suppression required a concentration of TOT approximately 1000 times greater than that required by the parental MCF-7 cells (i.e., 10^{-6} M). In contrast, the pure antiestrogen, ICI 164,384, showed similar dose-response curves for inhibition of E_2-stimulated growth in MCF-7 and MCF/TOT cells (Fig. 3, panel B).

Induction of pS2 mRNA, an early primary response to estrogen in MCF-7 cells [24], was used as an additional end-point to compare the ability of TOT to moderate E_2-stimulated responses in MCF/TOT vs parental MCF-7 cells. Interestingly, unlike proliferation, the dose response for TOT reversal of E_2-stimulated pS2 mRNA was similar in parent MCF-7 and MCF/TOT cells (Fig. 4). Also as shown in Fig. 4

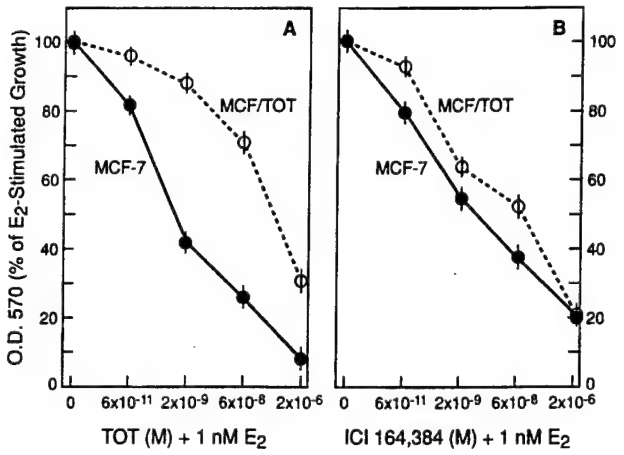

Fig. 3. Antiestrogen antagonism of E_2-stimulated growth. MCF-7 and MCF/TOT cells were seeded in quadruplicate at 2000 cells/well in 96-well plates and cotreated with 10^{-9} M E_2 and the indicated concentrations of TOT or ICI 164,384. Treatments were replenished on day 3 and cell number was determined by the MTT assay on day 6. E_2-stimulated growth was $953 \pm 50\%$ and $372 \pm 14\%$ of untreated, control cells in the parent MCF-7 and MCF/TOT cells, respectively. Values are expressed as percentage of absorbance in E_2-treated wells ($n = 4$; mean $\pm$ SEM).

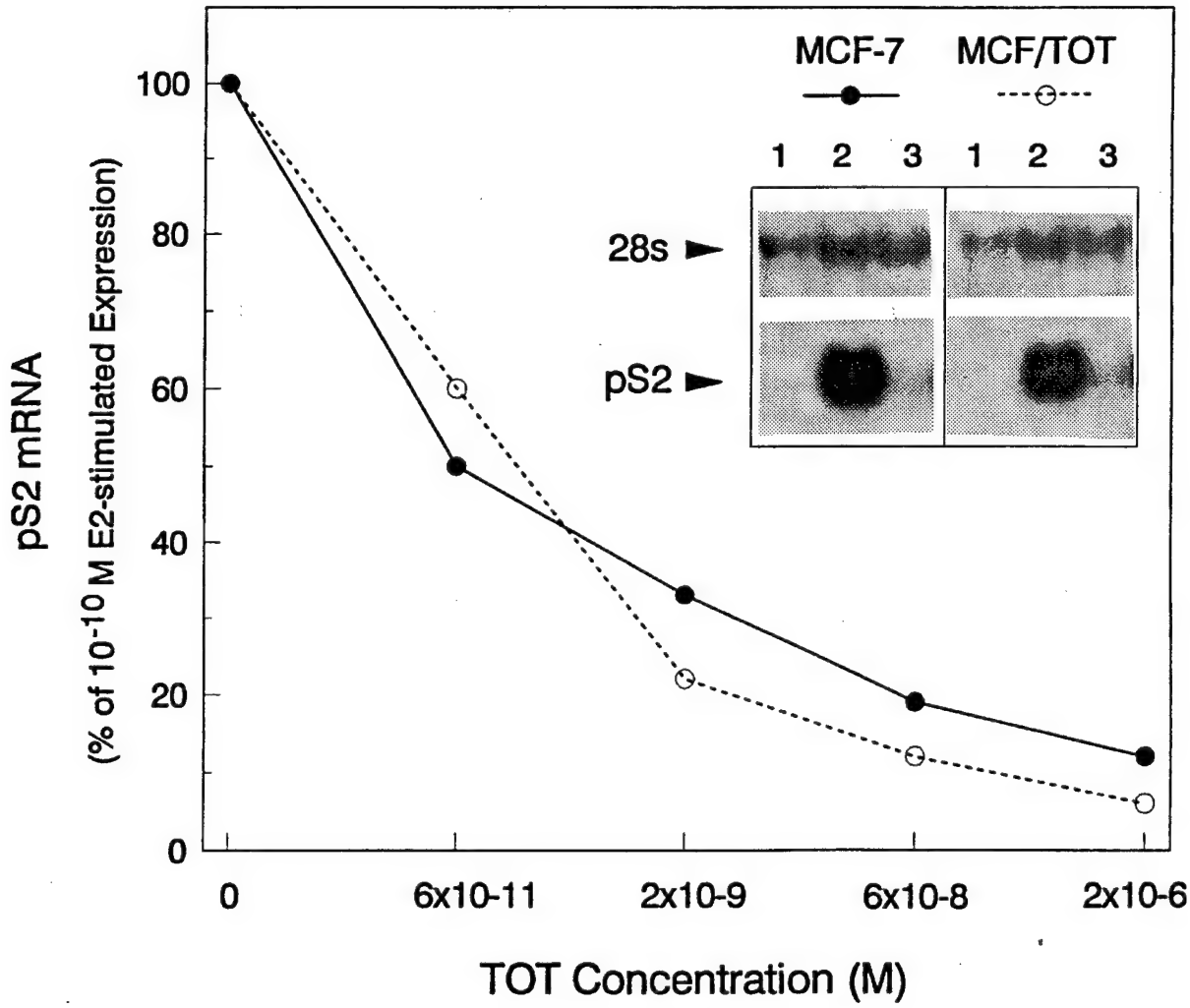

Fig. 4. Antagonism of E_2-stimulated pS2 mRNA expression by TOT. pS2 mRNA expression was analysed by Northern blot analysis of 20 μg of total RNA. Near confluent cell monolayers were treated with the ligands indicated for 12 h. Inset, autoradiogram of pS2 mRNA induction; lane 1, vehicle alone control; lane 2, 10^{-10} M E_2; lane 3, 10^{-6} M TOT.

(inset), pS2 was markedly stimulated by E_2, but showed no stimulation by TOT in either cell line. Therefore, TOT is not universally seen as an estrogen agonist for all responses in the MCF/TOT cells.

Additional markers of estrogen and antiestrogen responsiveness: regulation of progesterone receptor (PgR) and transactivation of an estrogen-responsive gene construct

Expression of PgR is known to be under tight estrogen regulation. In parent MCF-7 cells, treatment with 10^{-10} M E_2 resulted in a four-fold increase in PgR content (Table 1A~~, Table 2~~). In contrast, treatment of MCF/TOT cells with E_2 had no significant effect on PgR level (26.8 ± 2.2 vs 15.3 ± 5.2, respectively, $P > 0.05$). This was despite the presence of significant levels of ER in MCF/TOT cells, about half that present in the parent cell line (Fig. 5). A weak agonist effect of TOT was observed in parent MCF-7 cells in terms of PgR induction, but interestingly, TOT, like E_2, had no significant effect on PgR in the MCF/TOT subline (7.8 ± 2.2 vs 15.3 ± 5.2 fmol ^{3}H-R5020 bound/10^6 cells, respectively, $P > 0.05$). Both proliferation and PgR induction thus demonstrated altered regulation by E_2 and antiestrogen in MCF/TOT cells.

We also examined E_2 and antiestrogen responsiveness using another end-point, namely a transiently transfected estrogen-responsive gene construct containing a consensus estrogen response element (ERE) linked to a thymidine kinase (tk) promoter and the chloramphenicol acetyltransferase (CAT) gene (ERE-tk-CAT). In contrast to the loss of estrogen responsiveness of PgR in MCF/TOT cells, the transfected estrogen-responsive gene behaved similarly in parent MCF-7 and in MCF/TOT cells in terms of responsiveness to estrogen and antiestrogens. We observed comparable fold inductions of ERE-tk-CAT activity with 10^{-9} M E_2 in parent MCF-7 and MCF/TOT cells (Table 1B). TOT treatment did not significantly increase CAT activity in either subline, but it did substantially reverse the E_2-stimulated CAT activity. The response of ERE-tk-CAT was thus similar to that for induction of pS2 mRNA by estrogen and antiestrogen in that responses to these ligands were not altered in the MCF/TOT subline as compared to the parent MCF-7 cells.

Table 1. Markers of estrogen and antiestrogen responsiveness: regulation of progesterone receptor and transactivation of an estrogen-responsive gene construct

	fmol ^{3}H-R5020 bound/10^6 cells	
A	Parent MCF-7	MCF/TOT
Control vehicle	9.1 ± 3.3	15.3 ± 5.2
10^{-10} M E_2	43.4 ± 3.2*	26.8 ± 2.2
10^{-6} M TOT	26.8 ± 5.2*	7.8 ± 2.2

~~Table 2~~ omit

	Fold change in ERE-tk-CAT activity	
B	Parent MCF-7	MCF/TOT
Control vehicle	1.0 ± 0.2	1.0 ± 0.3
10^{-9} M E_2	11.5 ± 2.1*	8.2 ± 1.0*
10^{-6} M TOT	2.1 ± 0.5	0.8 ± 1.0
E_2 + TOT	3.1 ± 0.5*	2.3 ± 1.0
E_2 + ICI 164,384	0.5 ± 0.7	0.9 ± 0.7

A, Basal and stimulated progesterone receptor content was determined by binding of the progestin, [^{3}H]R5020, by whole cell hormone binding assay after 4 days treatment with ethanol vehicle control, 10^{-10} M E_2 or 10^{-6} M TOT. Values are the mean $\pm$ SEM of triplicate flasks from two experiments (*value significantly different from the control vehicle treated cells at $P < 0.05$ by Student's *t*-test). B, Transactivation of ERE-tk-CAT, a reporter plasmid containing a consensus estrogen response element linked to the Herpes simplex virus thymidine kinase promoter and the CAT reporter gene. ERE-tk-CAT (3 μg) was transiently cotransfected along with an internal control plasmid containing the lac-Z gene, and cells were treated with the ligands indicated for 24 h. The calculated fold increase in the CAT activity of each group was normalized for the β-galactosidase activity. Values are expressed as the mean $\pm$ SEM of at least three experiments (*value significantly different from the control vehicle treated cells at $P < 0.05$ by Student's *t*-test)..

Estrogen receptor content and regulation in MCF-7 and MCF/TOT cells

Estrogen receptor (ER) content was determined by whole cell binding assay and Western blot analysis. Parent MCF-7 cells contained 59.2 ± 4.6 fmol ER/10^6 cells (Fig. 5) and this level was stable throughout the time period of these experiments (data not shown). The MCF/TOT subline contained reduced levels of ER (28.7 ± 2.4 fmol ER/10^6 cells) at 50 weeks of maintenance in TOT (Fig. 5). This level of ER was maintained at 75 and 125 weeks of culture in TOT (34.1 ± 1.1 and 30.5 ± 1.3 fmol ER/10^6 cells, respectively). A comparable decrease in ER protein level in MCF/TOT cells was also observed when analysed by Western blot analysis ($37 \pm 6\%$ of parental level; Fig. 5).

We also used Western blot analyses to assess the ability of several agents to modulate the level of the ER protein. In both the parent MCF-7 cells and MCF/TOT cells, treatment with E_2 resulted in a marked (ca. 60%) decrease in ER protein level, whereas TOT treatment did not affect ER protein level or increased it slightly, and cotreatment of either subline with TOT prevented the decrease in ER protein content induced by treatment with E_2 alone (Fig. 5). Similar to E_2, treatment with retinoic acid (10^{-6} M) markedly decreased the ER level in both MCF-7 and MCF/TOT cells, and cotreatment with TOT prevented the reduction in ER seen in response to E_2 or retinoic acid treatment. ER level thus showed

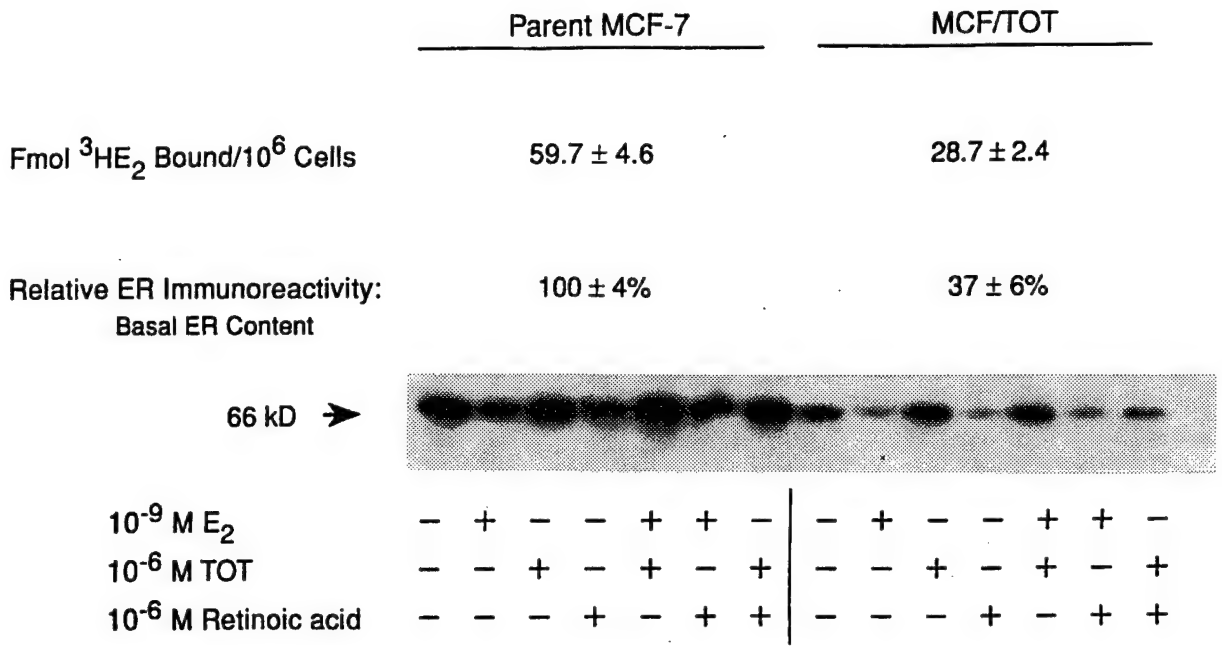

Fig. 5. Estrogen receptor (ER) content and effects of E_2, TOT and retinoic acid on ER levels in parent MCF-7 and MCF/TOT cells. Estrogen receptor content was determined by whole-cell hormone binding assay and Western blot analysis. For the whole-cell binding assay, cells in T25 flasks were incubated with 10 nM [^{3}H]E_2 in the absence or presence of a 100-fold excess of unlabelled ligand at 37°C for 40 min ($n = 3$; mean ± SEM). To measure immunoreactive ER, fractionated cellular protein was isolated from subconfluent T75 flasks treated with the indicated ligands for 24 h, as described in Materials and methods. ER protein was detected by binding of the ER-specific monoclonal antibodies H226 and D547. Detection of ER with the ER-specific antibody, H222 (with an exon 7 epitope), gave the same relative levels for the 66 kDa ER protein.

the same regulation by E_2, TOT and retinoic acid in parental MCF-7 and MCF/TOT cells.

Reversibility of the TOT growth-stimulated phenotype of MCF/TOT cells

To test whether the altered growth phenotype of the MCF/TOT cells was reversible, we removed TOT from the growth medium for a period of 16 weeks (Fig. 6, panel C) and compared growth response with that of the parent MCF-7 (Fig. 6, panel A) and MCF/TOT cells (Fig. 6, panel B). As a modification, we also generated another TOT-withdrawn subline which received high levels of E_2 (10^{-8} M) simultaneously with the TOT withdrawal for 16 weeks (Fig. 6, panel D). Interestingly, whereas the TOT-withdrawn subline was no longer growth stimulated by TOT, it did not revert to the TOT growth-inhibited phenotype of the parent MCF-7 cells (Fig. 6, panel A). Rather, this subline was refractory to the effects of 10^{-6} M TOT (Fig. 6, panel C; 104 ± 3% of control values). Similar results were obtained with the TOT-withdrawn, E_2-supplemented subline (111 ± 9% of control values). The TOT-withdrawn subline also exhibited a partial return to the relatively high ER levels of the parent MCF-7 cells (46.4 ± 0.3 vs 59.2 ± 4.6 fmol ER/10^6 cells, respectively) at 16 weeks of TOT deprivation. At 24 weeks of TOT

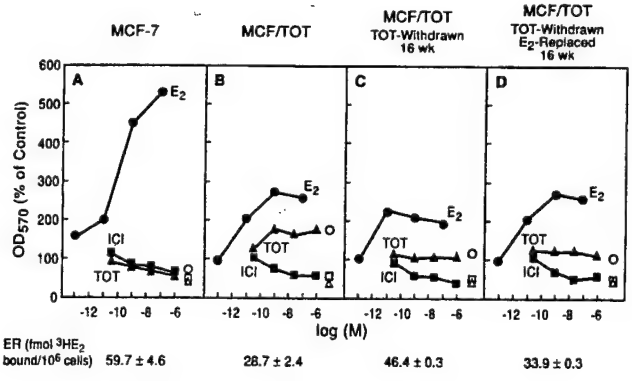

Fig. 6. Reversibility of the TOT growth-stimulated phenotype of the MCF/TOT cells. MCF/TOT cells were cultured in the absence of TOT with or without supplementation with 10^{-8} M E_2 for 16 weeks (panels C and D) and growth responses were compared with those of the parental MCF-7 (panel A) and MCF/TOT cells (panel B). Growth responsiveness to E_2, TOT and ICI 164,384, alone or in combination, was determined by MTT assay from quadruplicate wells. Closed circle, E_2; closed triangle, TOT; closed square, ICI 164,384; open circle, 10^{-9} M $E_2 + 10^{-6}$ M TOT; open triangle, 10^{-9} M $E_2 + 2 \times 10^{-6}$ M ICI 164,384; open square, 10^{-6} M TOT + 2×10^{-6} M ICI 164,384. Values are expressed as percentages of vehicle-treated control wells. Standard errors were less than 10% and are not shown. Estrogen receptor content was determined by whole-cell hormone binding assay ($n = 3$; mean ± SEM).

deprivation, there was no change in the proliferative profile of the sublines compared to the 16 week TOT-withdrawn cells; both were moderately growth stimulated by E_2, growth inhibited by ICI 164,384 and refractory to TOT (data not shown).

Estrogen receptor sequence analysis

To assess if alterations in ligand response in the MCF/TOT cells might be due to mutation of the ER, we amplified and sequenced a 1 kb region of the ER encompassing the hormone binding domain. Polymerase chain reaction yielded three cDNA products, which by direct sequence analysis were determined to be the wild type, exon 5 deletion variant (ΔE5) and the exon 7 deletion variant (ΔE7). The presence of these variants in breast cancers has previously been described [25, 26]. Dideoxy sequence analysis failed to reveal point mutations in the ERs from parental MCF-7 or MCF/TOT cells.

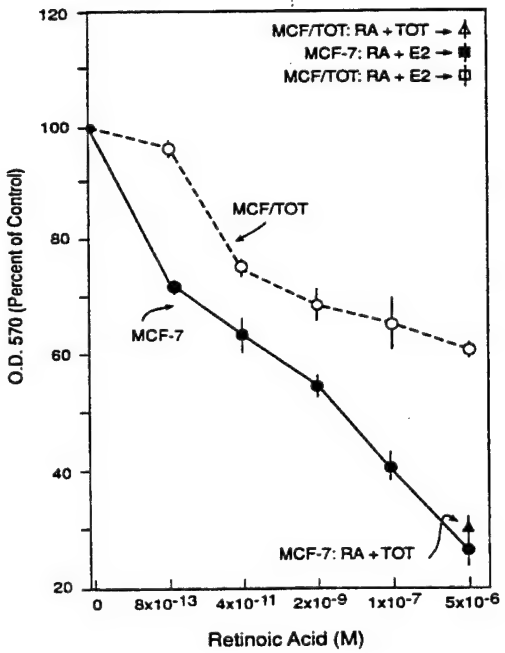

Fig. 7. Decreased responsiveness of MCF/TOT cells to the growth-inhibitory effects of retinoic acid. Cells were seeded at 2000 cells/well in 96-well plates in quadruplicate and treated with the indicated concentrations of retinoic acid for 6 days, with a media change after 3 days. Growth inhibition by retinoic acid was determined by MTT assay. The solid and open markers represent the parent MCF-7 and MCF/TOT cells, respectively; circles, retinoic acid treatment alone; squares, 5×10^{-6} M retinoic acid + 10^{-9} M E_2; triangles, 5×10^{-6} M retinoic acid + 10^{-6} M TOT. Values are expressed as the percentage of vehicle-treated control wells. Values for the retinoic acid dose-response curve represent the mean ± SEM of three separate experiments. Values for cotreatment with retinoic acid plus E_2 or TOT represent the mean ± range of two separate experiments.

Decreased responsiveness of MCF/TOT cells to the growth-inhibitory effects of retinoic acid

Retinoic acid analogues have been shown to inhibit the growth of a number of cancer cell lines, including MCF-7 cells [27]. To determine whether TOT-maintained MCF-7 cells differed in sensitivity to retinoic acid, we performed the dose-response growth study shown in Fig. 7. Parent MCF-7 cells were strongly growth inhibited by retinoic acid. Some growth suppression was observed even at very low concentrations of retinoic acid (8×10^{-13} M), and a growth suppression of approximately 75% was observed in MCF-7 cells at the highest concentration tested, 5×10^{-6} M retinoic acid. MCF/TOT cells were also sensitive to the growth suppressive effects of retinoic acid, albeit to a much lesser extent. MCF/TOT cells exhibited only 43 ± 2% growth suppression at 5×10^{-6} M retinoic acid. Cotreatment with retinoic acid and E_2 reversed the growth-suppressive effects of treatment with retinoic acid alone in both sublines (Fig. 7). Interestingly, whereas cotreatment with retinoic acid and TOT had no additional suppressive effect in parent MCF-7 cells (Fig. 7, filled triangle), TOT fully reversed the growth suppression by retinoic acid (Fig. 7, open triangle), indicating that TOT was acting as an agonist (stimulator) like E_2, in the MCF/TOT cells.

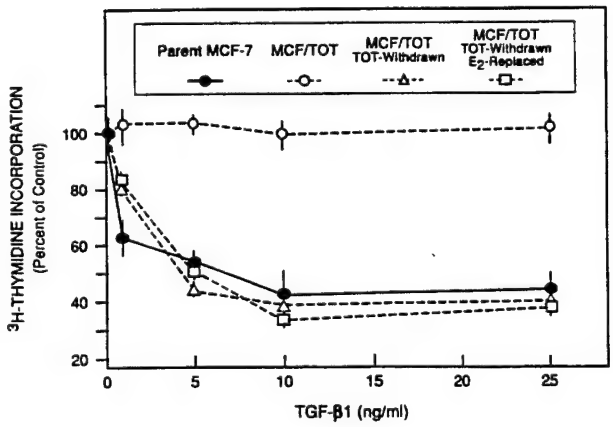

Fig. 8. Loss of growth inhibition by MCF/TOT cells in response to exogenous TGF-β1. Deprivation of TOT from MCF/TOT cells for 16 weeks, where indicated, was performed as described in the Materials and methods section. Cells were seeded at 2000 cells/well in triplicate in 24-well dishes. Two days later the wells were washed in serum-free media and then treated with TGF-β1 with or without 10^{-6} M TOT. After 4 days, the cells were incubated with 0.5 μCi [^{3}H]thymidine at 37°C for 2 h. Incorporated [^{3}H]thymidine was determined as described in Materials and methods. Basal [^{3}H]thymidine incorporation rates were comparable between the two sublines. Treatment with TGF-β1 in serum-supplemented or in serum-free IMEM yielded comparable results, as did measurement of cell number by MTT assay. Values are expressed as a percentage of vehicle-treated control wells (n = 3; SEM).

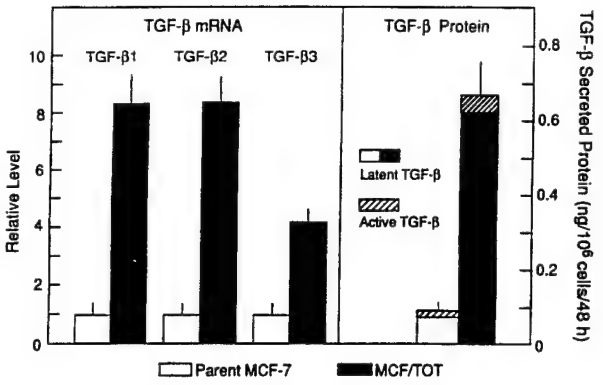

Fig. 9. Elevated TGF-β expression in MCF/TOT cells. TGF-β1, β2 and β3 mRNA expression in near-confluent cell cultures was determined by ribonuclease protection assay of 10 μg of total RNA, and normalized against human acidic phosphoprotein PO (36B4) as an internal control. RNase protection assays were quantitated by densitometric analyses of autoradiograms, as described in Materials and methods. Values represent the average and range of two experiments. Total and percentage active secreted TGF-β protein were determined from duplicate conditioned media collections by inhibition of [^{3}H]thymidine incorporation in Mv 1 Lu cells. Values represent the mean and range from the two separate experiments.

Loss of growth suppression by exogenous TGF-β1 in MCF/TOT cells

TGF-β1 is of interest due to its ability to inhibit the growth of human breast cancer cells [9]. Treatment with exogenous TGF-β1 resulted in dose-dependent decreases in [3H]thymidine incorporation in parent MCF-7 cells (Fig. 8). An inhibition of 40% was observed at 1 ng/ml TGF-β1, and a maximal inhibition of approximately 60% was observed at 5 or 10 ng/ml TGF-β1. Further suppression of growth was accomplished by cotreatment with TOT which resulted in an additional suppression of 20 ± 3% (data not shown). In contrast, [^{3}H]thymidine incorporation of MCF/TOT cells was unaffected by treatment with exogenous TGF-β1, even at 25 ng/ml. Sensitivity to TGF-β1 was re-established upon withdrawal of TOT from MCF/TOT cells. Removal of TOT from MCF/TOT cells for 16 weeks, either with or without supplementation with E_2, returned TGF-β1 sensitivity to that observed in the parent MCF-7 cells (Fig. 8).

Production of TGF-β mRNA and protein in MCF-7 and MCF/TOT cells

TGF-β mRNA level was monitored in parent MCF-7 and MCF/TOT cells by ribonuclease protection assay. As shown in Fig. 9, MCF/TOT cells expressed approximately eight-fold elevated levels of TGF-β1 and TGF-β2 mRNA, and approximately four-fold elevated levels of TGF-β3 mRNA, as compared to parent MCF-7 cells. The levels of bioactive TGF-β protein increased proportionally, as determined by a mink lung cell bioassay. No substantial changes in the proportion of latent and active secreted TGF-β were observed (Fig. 9). Therefore, MCF/TOT cells which no longer responded to the growth-regulating effects of exogenous TGF-β1 (Fig. 8), secreted elevated levels of TGF-β protein. We next sought to examine if the MCF/TOT cells showed alterations in TGF-β receptor expression.

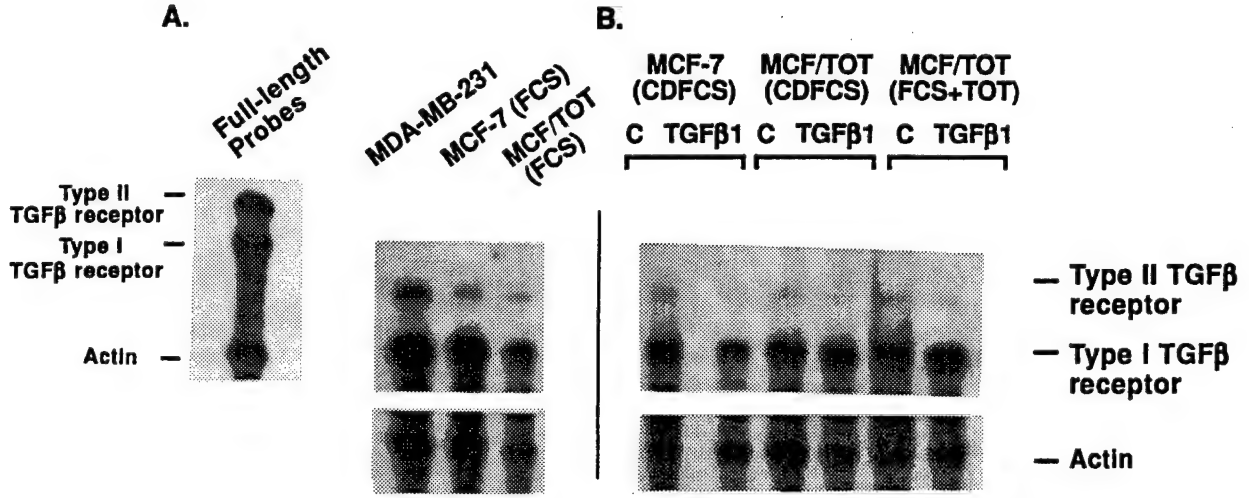

Fig. 10. Expression and ligand-induced regulation of type I and II TGF-β receptors. Total RNA from MCF-7 and MCF/TOT cells was isolated from subconfluent monolayers grown in 5% FCS MEM, with 10^{-6} M TOT where indicated, or in 5% CDFCS IMEM. Cells were treated without (C, control) or with 10 ng/ml TGF-β1 for 8 h. Thirty micrograms total RNA was hybridized with a 300 bp riboprobe for TGF-β type I receptor (220 bp protected fragment) and a 360 bp riboprobe for TGF-β type II receptor (260 bp protected fragment) and a 300 bp riboprobe for human β-actin (125 bp protected fragment), used as an internal control. RNase protection assays were performed and quantitated as described in Fig. 9 and the Materials and methods section. For comparison, the levels of type I and II TGF-β receptors in MDA-MB-231 breast cancer cells are shown.

Expression and ligand-induced regulation of type I and II TGF-β receptor mRNAs and assessment of TGF-β1 binding

Since TGF-β signals through a heteromeric complex of the type I and II TGF-β/activin receptors which possess serine-threonine kinase activity [28], we measured expression of type I and II TGF-β receptor mRNAs by ribonuclease protection assay (Fig. 10). There were no significant changes in the levels of these receptor mRNAs between the parent MCF-7 and MCF/TOT cells when lanes were normalized for the amount of RNA loaded. Furthermore, neither treatment with TGF-β1 for 8 h, nor transfer from full serum to steroid-depleted serum, influenced expression of these mRNAs. These results show that the loss of sensitivity of the MCF/TOT cells to the growth-inhibitory effects of exogenous TGF-β can not be attributed to loss of expression of type I or II TGF-β receptors. We also performed [^{125}I]TGF-β1 binding assays to confirm that the receptor moieties present were functionally able to bind exogenous TGF-β1. We found 282 ± 30 ($n = 3$) [^{125}I]TGF-β1 binding sites/cell in the parent MCF-7 cells. The MCF/TOT cells showed an approximate three-fold increase in the number of TGF-β1 binding sites per cell (949 ± 102, $P < 0.05$). Therefore, the loss of growth-inhibitory response to exogenous TGF-β1 by MCF/TOT cells is not due to a decrease in TGF-β1 binding sites.

DISCUSSION

This report describes a new subline of MCF-7 cells which, in response to long-term exposure to TOT, developed resistance to the growth-inhibitory effects of this antiestrogen and also altered sensitivity to the growth-suppressive effects of exogenous TGF-β1 and retinoic acid. Furthermore, the weak stimulation of MCF/TOT cell proliferation by TOT implies that growth resistance in these cells really corresponds to a weak growth stimulation by this agent. Interestingly, these MCF/TOT cells were still responsive to suppression by the pure antiestrogens ICI 164,384 and ICI 182,740, implying that these two categories of antiestrogens must act, at least in part, by somewhat different mechanisms. Although one proposed mechanism of antiestrogen resistance is loss or mutation of estrogen receptor [29–32], our observation that the phenotype of the MCF/TOT cells is at least partly reversible following withdrawal from TOT implies a non-mutational change in these cells, consistent with our observation that ER in the parental and MCF/TOT cells had identical hormone-binding domains, as determined by DNA sequencing analysis.

Response-specific antiestrogen resistance

Whereas tamoxifen is associated with growth inhibition of breast tumors, it is also a cell- and promoter-dependent agonist. Tamoxifen shows tissue- and gene-specific estrogen-like effects, being a good estrogen agonist in bone and uterine cells and a good stimulator of some, but not all, estrogen-regulated genes [7, 33]. The ER is now known to interact with multiple proteins, termed coactivators and corepressors (reviewed in [34]), that contact different regions of the ER and can influence ER transcriptional activity greatly. Differences in the interaction of antiestrogen-ER complexes with coactivators and corepressors in different cells and at different gene sites could account for the cell- and gene-selective actions of antiestrogens in parental ER-positive breast cancer cells and in our breast cancer cells selected for resistance to growth suppression by TOT. It is perhaps to be expected, as we have observed in the present studies, that the alteration in TOT-response profile of MCF/TOT cells varied with the end-point monitored. Whereas TOT behaved agonistically in terms of proliferation in the MCF/TOT subline, there was a complete loss of its partial agonistic effects on induction of progesterone receptor expression (Table 1). Interestingly, estrogen also failed to increase progesterone receptor in this subline, as reported in other tamoxifen-resistant breast cancer cells [32, 35]. We found, however, that the usual stimulatory and inhibitory effects of E_2 and TOT, respectively, were maintained in terms of regulation of pS2 mRNA induction and ERE-tk-CAT transactivation. These results demonstrate that loss of TOT growth inhibition is not synonymous with a global loss of responsiveness to TOT. Other MCF-7 cell variants which were tamoxifen-stimulated in terms of growth also did not exhibit corresponding tamoxifen stimulation of the estrogen-regulated mRNAs pNR-1, -2, -25, and cathepsin-D [36].

In the present work, the growth of MCF/TOT cells was dramatically suppressed by treatment with the pure antiestrogen, ICI 164,384, and this antiestrogen antagonized the effects of either E_2 or TOT on growth and gene regulation in MCF/TOT cells. ICI 164,384 has been shown to block ER action by accelerating ER degradation [37, 38] as well as inefficiently promoting transcription activation [38]. Unlike ICI 164,384, TOT treatment does not decrease ER protein content (Fig. 5; and [38]). These results, as well as the observed beneficial response to the ICI 164,384-related pure antiestrogen ICI 182,780 in tamoxifen-resistant breast cancers in women [39] and nude mouse tumor models [40, 41], support the potential clinical use of ICI 164,384-type antiestrogens in the advent of tamoxifen resistance.

Structure of the estrogen receptor

Whereas it seems plausible that mutations in the ER gene could affect ligand interpretation by the ER, our finding that TOT-stimulated growth in MCF/TOT cells is partly reversible upon withdrawal of TOT for a period of 16 weeks suggests that a readily modifiable process, rather than a mutational event, is responsible for the antiestrogen insensitivity. Alternate splicing of the ER mRNA into receptor species with different functions would allow for modulation of the receptor protein, without gene mutation. A number of ER variant mRNAs are expressed in breast neoplasms and some of these variants have been found to possess either constitutively active or inhibitory receptor activity [25, 42].

Our analysis of the nucleotide sequence of the hormone-binding domain of the ER revealed the presence of wild-type and exon 5 and exon 7 deletion variants, but failed to detect any mutations or other splicing variants in the parent MCF-7 and MCF/TOT sublines. Analysis of the ERs of other hormone-resistant sublines of MCF-7 or T47D human breast cancer cells by RNase protection mapping [43] or PCR amplification [44] also failed to detect variants or mutants of the ER. Recently, Karnik *et al.* [45] screened 20 tamoxifen-sensitive and 20 tamoxifen-resistant human breast tumors by single-strand conformation polymorphism and found ER mutations were neither frequent nor correlated with an antiestrogen-resistant phenotype. The altered hormonal responsiveness seen in MCF/TOT cells is thus unlikely to be due to mutational change in the ER.

Cross-talk with retinoids and transforming growth factor-β in the antiestrogen resistance of MCF/TOT cells

The antiestrogenic character of the retinoids has implicated them as candidates for combination palliative therapy in ER-containing breast cancers. We found that our MCF/TOT cells exhibited decreased sensitivity to retinoic acid. This may be explained by the fact that retinoids, which have been shown to modulate estrogenic regulation of a number of mRNAs, including those for pS2 and the growth-stimulator TGF alpha [9], are thought to exert their growth-inhibitory effects through the ER as well as their own receptors [10, 27, 46]. Therefore, the reduced retinoic acid-induced growth suppression we observed could be, at least in part, due to the reduced levels of ER present in the MCF/TOT subline as compared to parent MCF-7 cells. This would be consistent with recent observations that the introduction of ER into ER-negative breast cancer cells re-establishes retinoic acid growth inhibition [10].

We examined TGF-β production and TGF-β receptors in our parental and MCF/TOT cells because expression of TGF-β is known to be significantly influenced by sex steroid hormones [47–50]. Because TGF-β1 was a good growth inhibitor in our parental MCF-7 cells (Fig. 8), TGF-β resistance might thwart the suppressive, beneficial actions of tamoxifen. We observed that the MCF/TOT subline was resistant to the growth-inhibitory effects of exogenous TGF-β1 and that this insensitivity to added TGF-β1 was reversible following withdrawal of TOT. We also failed to observe a decrease in the expression of type I or II TGF-β receptor mRNAs or a decrease in the binding of [^{125}I]TGF-β1 in MCF/TOT cells. The TGF-β receptor system is highly complex, however, and includes at least one other characterized protein, the type III TGF-β receptor, and numerous receptors with TGF-β cross-reactivity [28] which were not evaluated in the present work.

Of note, MCF/TOT cells showed elevated production of TGF-βs. The cells contained eight times more TGF-β1 and TGF-β2 mRNAs and four times more TGF-β3 mRNA. They secreted three times more TGF-β bioactive protein and eight times more total (latent plus active) TGF-β protein than parental MCF-7 cells. Therefore, we do not know if their insensitivity to added TGF-β1 was due to the high level of TGF-β production possibly resulting in the generation of maximum autocrine TGF-β activity. We think this is unlikely, however, because it is worth noting that MCF/TOT cells grow very quickly (ca. 1.6 day doubling time) in the presence of TOT and therefore are not being growth suppressed by the TGF-βs either being made and secreted by the cells, or by the TGF-β1 we added exogenously. In addition, we previously reported that short-term estrogen-deprived MCF-7 cells contained 10 times more TGF-β1 mRNA, eight times more TGF-β2 mRNA and five times more TGF-β3 mRNA, and secreted four times more bioactive TGF-β and three times more total (active plus latent) TGF-β than parental MCF-7 cells, yet these cells still showed normal, i.e. full, sensitivity to growth suppression by added TGF-β1 [20]. More detailed analyses of the TGF-β pathway in the MCF/TOT cells will be needed to understand fully the changes induced by antiestrogen exposure.

Our findings highlight the response-specific nature of antiestrogen resistance in breast cancer cells. To our knowledge, this is the first study to compare responses to antiestrogens and to the growth-inhibitory factors retinoic acid and TGF-β in breast cancer cells selected for resistance to tamoxifen. The reduced sensitivity to these agents in the MCF/TOT cells, and the restoration of responsiveness to these agents after TOT withdrawal suggests a possible commonality of components or pathways in their regulation of proliferation of these human breast cancer cells. Our findings indicate also that one mechanism of antiestrogen resistance, as seen in MCF/TOT cells, may involve alterations in growth factor and other hormonal pathways that affect the ER response pathway.

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2 Estrogen–Receptor and Antiestrogen–Receptor Complexes: Cell- and Promoter-Specific Effects and Interactions with Second Messenger Signaling Pathways

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2.1 Introduction and Overview

Estrogens regulate the differentiation, growth, and functioning of many reproductive tissues. They also exert important actions on other tissues, including bone, liver, and the cardiovascular system. Most of the actions of estrogens appear to be exerted via the estrogen receptor (ER) of target

cells, an intracellular receptor that is a member of a large superfamily of proteins that function as ligand-activated transcription factors, regulating the synthesis of specific RNAs and proteins. Because of this diversity of estrogen target tissues, much current interest focuses on trying to understand the basis for the cell and promoter context-dependent actions of estrogens and antiestrogens. Antiestrogens, which antagonize the actions of estrogens, have much potential as important therapeutic agents. Although antiestrogens bind to the ER in a manner that is competitive with estrogen, they fail to effectively activate gene transcription (Jordan and Murphy 1990; Katzenellenbogen et al. 1985; Santen et al. 1990). Two of the major challenges in studies on antiestrogens are to understand what accounts for their antagonistic effectiveness, as well as the partial agonistic effects of some antiestrogens, and to understand how one can attain tissue-selective agonist/antagonist effects of these compounds.

In order to address these issues, many of our analyses have focused in detail on the hormone-binding domain of the ER, regions E and F, since this domain of the receptor contains both hormone-binding and hormone-dependent transactivation functions of the receptor. In our attempts to understand how the ER discriminates between estrogen and antiestrogen ligands, and between different categories of antiestrogens, we have generated and analyzed variant human ERs with mutations in the ER hormone-binding domain and studied the activity of these receptors on different estrogen-responsive genes in several cell backgrounds when liganded with antiestrogenic or estrogenic ligands. These studies, and those of others, have provided consistent evidence for the promoter-specific and cell-specific actions of the estrogen-occupied and antiestrogen-occupied ER. In addition, although the binding of estrogens and antiestrogens is mutually competitive, studies with ER mutants indicate that some of the contact sites of estrogens and antiestrogens are likely different. Our recent studies reveal that the presence of the C-terminal F domain of the ER is important in the transcription activation and repression activities of antiestrogens and that it affects the magnitude of liganded ER bioactivity in a cell-specific manner (Montano et al. 1994, 1995). The influence of the F domain on the agonist/antagonist balance and potency of antiestrogens supports its specific modulatory role in the ligand-dependent interaction of ER with components of the transcription complex. These studies (Ince et al. 1993, 1995; Katzenellenbogen et al. 1993; Pakdel and Katzenellenbogen 1992; Wrenn and Katzenellenbogen 1993, see below) have provided evidence for a regional dissociation of the hormone-binding and transcription-activation regions in domain E of the receptor and have also shown that mutations in the hormone-binding domain and deletions of C-terminal regions result in ligand discrimination mutants, that is, receptors that are differentially altered in their ability to bind and/or mediate the actions of estrogens versus antiestrogens.

In addition, in studies described below, we have observed that protein kinase activators enhance the transcriptional activity of the ER and alter the agonist/antagonist balance of some antiestrogens, suggesting that changes in the cellular phosphorylation state should be important in determining the effectiveness of antiestrogens as estrogen antagonists. The ability of estrogens and antiestrogens to also increase cAMP levels in target cells suggests that the interaction of estrogens with second messenger signaling pathways may be bidirectional.

2.2 Estrogen and Antiestrogen Binding and Discrimination by the ER

A variety of studies (Berry et al. 1990; Fujimoto and Katzenellenbogen 1994; Pakdel et al. 1993a, b; Pakdel and Katzenellenbogen 1992; Reese and Katzenellenbogen 1991, 1992a, b; Tzukerman et al. 1994; Wrenn and Katzenellenbogen 1993) have provided strong documentation that the response of genes to estrogen and antiestrogen depend on several important factors: (a) the nature of the ER, i.e., whether it is wild type or variant; (b) the ligand; (c) the promoter; and (d) the cell context. The gene response, in addition, can be modulated by cAMP, growth factors, and agents that affect protein kinases and cell phosphorylation (Aronica and Katzenellenbogen 1991, 1993; Cho et al. 1994; Fujimoto and Katzenellenbogen 1994; Kraus et al. 1993). These factors, no doubt, account for differences in the relative agonism/antagonism of antiestrogens, for instance, tamoxifen, on different genes and in different target cells such as those in breast cancer cells versus uterine or bone cells.

Although both estrogens and antiestrogens bind within the hormone-binding domain, the association must differ because estrogen binding

activates a transcriptional enhancement function, whereas antiestrogens fully or partially fail in this role. Antiestrogens are believed to act in large measure by competing for binding to the ER and altering the conformation of the ER such that the receptor fails to effectively activate gene transcription. In addition, antiestrogens exert anti-growth factor activities via a mechanism that requires ER but is still not fully understood (Freiss and Vignon 1994). Models of antiestrogen action at the molecular level are beginning to emerge, and recent biological studies also indicate that antiestrogens fall into at least two distinct categories: antiestrogens such as tamoxifen that are mixed or partial agonists/antagonists (type I), and compounds such as ICI164,384 that are complete/pure antagonists (type II). The type I antihormone–ER complexes appear to bind as dimers to EREs; there, they block hormone-dependent transcription activation mediated by region E of the receptor, but are believed to have little or no effect on the hormone-independent transcription activation function located in region A/B of the receptor (Berry et al. 1990). Thus, they are generally partial or mixed agonist/antagonists, and their action must involve some subtle difference in ligand–receptor interaction very likely associated with the basic or polar side chain that characterizes the antagonist members of this class. In the case of the more complete antagonists such as ICI164,384, ER conformation must clearly differ from that of the estrogen-occupied ER since alteration in ER binding to DNA and reduction of the ER content of target cells appear to contribute to (Dauvois et al. 1992; Fawell et al. 1990b), but may not fully explain, the pure antagonist character of this antiestrogen (Reese and Katzenellenbogen 1992b). Of note is the fact that antiestrogens, whether steroidal or nonsteroidal, typically have a bulky side chain which is basic or polar. This side chain is important for antiestrogenic activity; removal of this side chain results in a compound which is no longer an antiestrogen and, instead, has only estrogenic activity. Therefore, interaction of this side chain with the ER must play an important role in the interpretation of the ligand as an antiestrogen.

In order to understand how the ER "sees" an antiestrogen as different from an estrogen, we have used site-directed and random chemical mutagenesis of the ER cDNA to generate ERs with selected changes in the hormone-binding domain. We have been particularly interested in identifying residues in the hormone-binding domain important for the

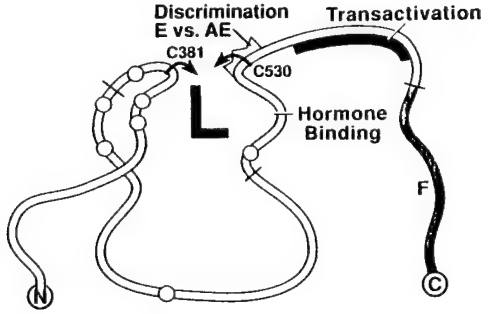

Fig. 1. "Map" of functions in the human estrogen receptor hormone-binding domain (HBD). Domain E, amino acids 302-553, is shown as is the very C-terminal domain *F*, amino acids 554-595. Some regions considered to be important in hormone binding, discrimination between estrogen (*E*) and antiestrogen (*AE*), and transactivation are highlighted. The ligand (*L*) is portrayed in a region representing the ligand binding pocket of the receptor. *Open circles* indicate amino acids in the HBD where our mutational analyses have shown mutational changes to affect the affinity or stability of hormone binding. See text for description. *N*, N-terminus; *C*, C-terminus of receptor

ligand binding of estrogen and antiestrogen and transactivation functions of the receptor and in elucidating the mechanism by which the ER differently interprets agonistic and antagonistic ligands. Our studies have indicated that selective changes near amino acid 380 and amino acids 520-530 and changes at the C-terminus of the ER result in ER ligand discrimination mutants (Montano et al. 1994, 1995; Pakdel et al. 1993b; Pakdel and Katzenellenbogen 1992). These data provide evidence that some contact sites of the receptor with estrogen and antiestrogen differ and that the conformation of the receptor with estrogen and antiestrogen must also be different as a consequence (Danielian et al. 1993; Pakdel and Katzenellenbogen 1992, references therein).

Our structure–function analysis of the hormone-binding domain of the human ER has utilized region-specific mutagenesis of the ER cDNA and phenotypic screening in yeast, followed by the analysis of interesting receptor mutants in mammalian cells (Katzenellenbogen et al. 1993; Wrenn and Katzenellenbogen 1993). A great advantage of the yeast system is that it allows the rapid screening of a library of many mutants, a situation that is not possible in mammalian cells. Our obser-

vations, as well as very important studies by Malcolm Parker and colleagues (Danielian et al. 1992, Fawell et al. 1990a), have shown a separation of the transactivation and hormone-binding functions of the ER, with amino acids critical in the transactivation function of the receptor being more C-terminal in domain E (see Fig. 1). Interestingly, some transcriptionally inactive receptors with modifications in this domain E C-terminal activation function 2 (AF-2) region of the ER have potent dominant negative activity, being able to suppress the activity of the wild-type ER in cells (Ince et al. 1993, 1995).

Since the basic or polar side chain is essential for antiestrogenic activity, and our previous studies identified cysteine 530 as the amino acid covalently labeled by affinity-labeling ligands (Harlow et al. 1989), we introduced by site-directed mutagenesis of the ER cDNA changes of specific charged residues close to C530 (Pakdel and Katzenellenbogen 1992). Interestingly, two mutants in which lysines at position 529 and 531 were changed to glutamines, so that the local charge was changed, resulted in ligand discrimination mutants. These receptors showed an approximately thirtyfold increased potency of antiestrogen in suppressing estradiol (E_2)-stimulated reporter gene activity. Interestingly, these mutant receptors had a reduced binding affinity for estrogens, but retained unaltered binding affinity for antiestrogen. These observations suggest that we are able to differentially alter estrogen and antiestrogen effectiveness by rather modest changes in the ER, and that the region near C530 is a critical one for sensing the fit of the side chain of the estrogen antagonist. Studies from the Parker Laboratory (Danielian et al. 1993) have shown that nearby residues (i.e., G525 and M521 and/or S522 in the mouse ER) are also importantly involved in conferring differential sensitivity to these two categories of ligands.

2.3 The Carboxy-Terminal F Domain of the ER: Role in the Transcriptional Activity of the Receptor and the Effectiveness of Antiestrogens as Estrogen Antagonists in Different Target Cells

Among the nuclear hormone receptors, ER is unusual in having a large C-terminal F region (42 amino acids) and its role in ER bioactivity and in the activities of antiestrogens has not been well defined. Previous studies by us and others have shown that domain F is not required for transcriptional response to E_2 (Kumar et al. 1986, 1987; Pakdel et al. 1993a), and, in addition, our studies have shown that this region does not affect the turnover rate of ER in target cells (Pakdel et al. 1993a). However, a more complete examination (Montano et al. 1994, 1995) has revealed that the presence of the F domain is important in the transcription activation and repression activities of antiestrogens, and that it affects the magnitude of liganded ER bioactivity in a cell-specific manner. Thus, in ER-negative breast cancer cells and Chinese hamster ovary (CHO) cells, E_2 stimulated equally the transcription of several estrogen-responsive promoter-reporter gene constructs with wild-type ER, and with ER lacking the carboxy-terminal F domain or ER altered in the F domain by point mutations. Of interest, the antiestrogens trans-hydroxytamoxifen and ICI164,384, which showed considerable agonistic activity on some of the reporter constructs with the wild-type ER, showed no agonistic activity with the ER lacking the F domain. In addition, the antiestrogens were more potent antagonists of E_2-stimulated transcription by the F domain-deleted ER than by wild-type ER. Interestingly, the effect of the F domain was very dependent on the cell type. In HeLa human cervical cancer cells, the F domain-deleted ER exposed to E_2 was much less effective than wild-type ER in stimulating transcription, and antiestrogens were less potent in suppressing E_2-stimulated transcription by the F domain-deleted ER. Since we find that the F domain does not appear to affect estrogen or antiestrogen ligand-binding affinity or DNA binding of the receptor, the fact that this region makes the liganded ER either more or less transcriptionally effective in different cells suggests that it plays an important role in maintaining ER conformation optimal for protein–protein interactions needed for transcriptional effectiveness. Its influence on the agonist/antagonist balance

and potency of antiestrogens further supports its specific modulatory role in the ligand-dependent interaction of ER with components of the transcription complex. Therefore, these studies (Montano et al. 1994, 1995) as well as several others by us (Cho and Katzenellenbogen 1993; Fujimoto and Katzenellenbogen 1994; Katzenellenbogen et al. 1995) and those of others have highlighted that beyond differences in estrogen and antiestrogen binding to the ER, the cell context and promoter context of the ER influences the estrogenic/antiestrogenic activity of antiestrogens.

2.4 Cross-Talk Between the ER and Second Messenger Signaling Pathways in Cells

Evidence for cross-talk between steroid hormone receptors and signal transduction pathways has been increasing. Expression of AP-1, a transcription factor of the Fos/Jun heterodimer known to mediate the protein kinase-C (PKC) pathway (Angel et al. 1987), was shown to suppress steroid hormone receptor-mediated gene expression (Schüle et al. 1990), most likely through direct protein–protein interaction between steroid receptors and these oncoproteins (Yang-Yen et al. 1990). In addition, the ovalbumin gene promoter containing a half-palindromic estrogen-responsive element (ERE) was coactivated by ER and Fos/Jun oncoproteins (Gaub et al. 1990). Thus, interaction between these oncoproteins and steroid hormone receptors resulted in cell-specific inhibitory or stimulatory effects on transcriptional activation (Gaub et al. 1990; Shemshedini et al. 1991; Strähle et al. 1988; Yang-Yen et al. 1990).

Previous studies by us and others (Aronica and Katzenellenbogen 1991; Katzenellenbogen and Norman 1990; Sumida et al. 1988; Sumida and Pasqualini 1989, 1990) documented upregulation of intracellular progesterone receptor, an estrogen-stimulated protein, by insulin-like growth factor-I (IGF-I), epidermal growth factor, phorbol ester, and cAMP in MCF-7 human breast cancer cells and uterine cells. The fact that the stimulation by these diverse agents was blocked by antiestrogen suggested that these agents were presumably acting through the ER pathway (Aronica and Katzenellenbogen 1991; Katzenellenbogen and Norman 1990; Sumida et al. 1988; Sumida and Pasqualini 1989). In addition, the fact that protein kinase inhibitors also blocked the effects of estrogen, cAMP, and growth factors suggested the involvement of phosphorylation in these responses. We therefore undertook studies to examine directly whether activators of protein kinases can modulate transcriptional activity of the ER.

In primary cultures of uterine cells using transient transfection experiments with simple estrogen-responsive reporter genes, we examined the ability of these agents to stimulate ER-mediated gene transcription and also compared the ability of these multiple agents to alter the phosphorylation state of the endogenous uterine ER protein. The results of our study (Aronica and Katzenellenbogen 1993) indicate that E_2, IGF-I, and agents which raise intracellular cAMP are able to stimulate ER-mediated transactivation and ER phosphorylation. The fact that antiestrogen (ICI164,384) evokes a similar increase in ER phosphorylation without a similar increase in transcription activation indicates that an increase in overall ER phosphorylation does not necessarily result in increased transcriptional activity. Also, the observation that transcriptional activation by the ER was nearly completely suppressed by the protein kinase inhibitors H8 and PKI, while the increase in phosphorylation was reduced by 50%–75%, indicates that the correlation between transcriptional activation and overall ER phosphorylation is not direct but does suggest that some of the effects of E_2, IGF-I and cAMP on ER-regulated transactivation are mediated through the activity of protein kinases. Our findings, demonstrating a clear effect of these agents on ER-mediated transactivation, suggest that these agents might also regulate endogenous estrogen target genes, such as that encoding the progesterone receptor, by similar cellular mechanisms.

In order to examine some of the molecular mechanisms controlling transcription of the progesterone receptor gene, we cloned the rat progesterone receptor gene 5'-region and identified two functionally distinct promoters (Kraus et al. 1993). The two distinct promoters in the rat progesterone receptor gene exhibited differential responsiveness to E_2 and to ER-dependent stimulation by cAMP. The functional differences between these two promoters may lead to altered expression of the A and B progesterone receptor isoforms and, thereby, influence cellular responsiveness to progestins (Kraus et al. 1993).

In MCF-7 human breast cancer cells and other cells, we found that activators of PKA and PKC markedly synergize with E_2 in ER-mediated

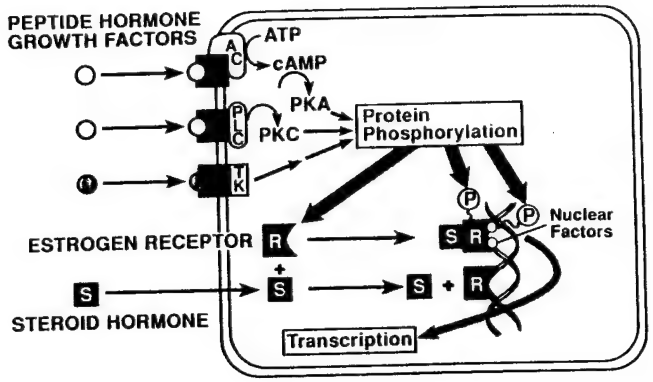

Fig. 2. Protein kinase–estrogen receptor transcriptional synergism. See text for description. *AC*, adenylate cyclase; *PLC*, phospholipase C; *TK*, tyrosine kinase; *PKC*, protein kinase C; *PKA*, protein kinase A; *R*, estrogen receptor; *S*, steroid hormone; *P*, phosphate groups on proteins

transcriptional activation and that this transcriptional synergism shows cell and promoter specificity (Cho and Katzenellenbogen 1993; Fujimoto and Katzenellenbogen 1994; Kraus et al. 1993). The synergistic stimulation of ER-mediated transcription by E_2 and protein kinase activators did not appear to result from changes in ER content or in the binding affinity of ER for ligand or the ERE DNA, but rather may be a consequence of a stabilization or facilitation of interaction of target components of the transcriptional machinery, possibly either through changes in phosphorylation of ER or other proteins important in ER-mediated transcriptional activation (Cho and Katzenellenbogen 1993).

Figure 2 shows a model indicating how we think the protein kinase-ER transcriptional synergism might occur. Agents influencing protein kinase pathways may enhance intracellular protein phosphorylation resulting in either phosphorylation of the ER itself or the phosphorylation of nuclear factors with which the receptor interacts in mediating transcription. Likewise, there is evidence that the steroid hormone itself can alter receptor conformation increasing its susceptibility to serve as a substrate for protein kinases (Ali et al. 1993; Aronica and Katzenellenbogen 1993; Denton et al. 1992; Lahooti et al. 1994; Le Goff et al. 1994). Therefore, agents which increase phosphorylation may, either

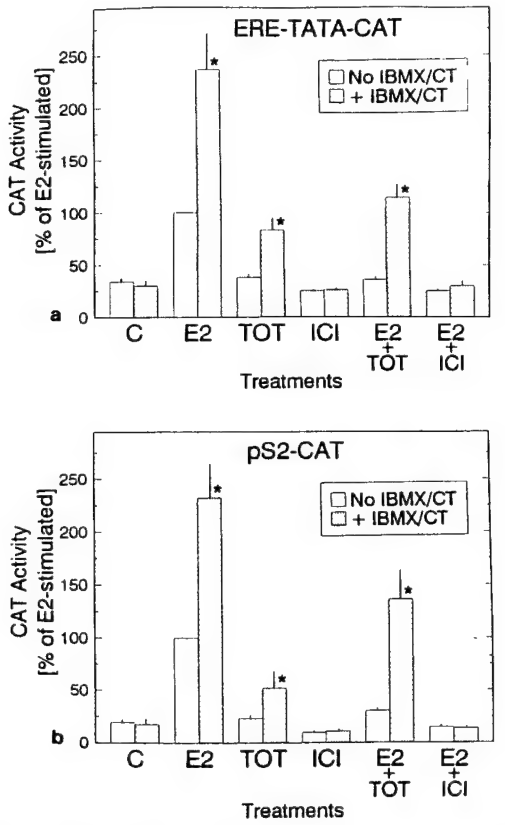

Fig. 3a,b. Effect of IBMX/CT on the ability of E_2 and antiestrogens to stimulate transactivation of ERE-TATA-CAT (**a**) and pS2-CAT (**b**), and on the ability of antiestrogens to suppress E_2-stimulated transactivation. MCF-7 cells were transfected with the indicated reporter plasmid and an internal control plasmid that expresses β-galactosidase and were treated with the agents indicated for 24 h. Each *bar* represents the mean ± SEM (n=3 experiments). * indicates significant difference from the no IBMX/CT cells ($p < 0.05$). *C*, control ethanol vehicle; E_2, 10^{-9} *M*; *TOT*, *trans*-hydroxytamoxifen (10^{-6} *M*); *ICI*, ICI 164384 (10^{-6} *M*); *IBMX*, 3-isobutyl-1-methyl-xanthine (10^{-4} *M*); *CT*, cholera toxin (1 μg/ml). (From Fujimoto and Katzenellenbogen 1994)

through phosphorylation of the ER itself or through phosphorylation of nuclear factors required for ER transcription, result in synergistic activation of ER-mediated transcription.

As shown in Fig. 3, we have compared the effects of cAMP on the transcriptional activity of E_2 liganded and antiestrogen-liganded ER complexes. We find that increasing the intracellular concentration of cAMP, or of protein kinase A catalytic subunits by transfection (Fujimoto and Katzenellenbogen 1994), activates and/or enhances the transcriptional activity of type I but not type II antiestrogen-occupied ER complexes and reduces the estrogen antagonist activity of the type I trans-hydroxy-tamoxifen (TOT) antiestrogen. In Fig. 3a,b, we have determined in MCF-7 human breast cancer cells the effect of cAMP on the activity of TOT, ICI164,384, and E_2 on a simple TATA promoter with one consensus ERE upstream of the CAT gene and on the more complex pS2 gene promoter and 5' flanking region (–3000 to +10) containing an imperfect ERE. The endogenous pS2 gene is regulated by E_2 in MCF-7 breast cancer cells. E_2 increased the transcription of both of these gene constructs, and treatment with isobutyl-methyl-xanthine and cholera toxin (IBMX/CT) and E_2 evoked a synergistic increase in transcription, with activity being ca. 2.5 times that of E_2 alone. Both antiestrogens (TOT and ICI) failed to stimulate transactivation of these reporter gene constructs, but in the presence of IBMX/CT, TOT gave significant stimulation of transcription (85% or 60% that of E_2 alone). ICI failed to stimulate transactivation even in the presence of IBMX/CT, and ICI fully blocked E_2 stimulation in the presence or absence of cAMP. By contrast, treatment with IBMX/CT reduced the ability of TOT to inhibit E_2 transactivation. While TOT returned E_2 stimulation down to that of the control in the absence of IBMX/CT (compare open bars E_2 vs. E_2+TOT in Fig. 3), TOT only partially suppressed the E_2 stimulation in the presence of IBMX/CT (compare stippled bars E_2 vs. E_2+TOT in Fig. 3).

Although alteration in the agonist and antagonist activity of TOT was observed with promoter–reporter constructs containing a simple TATA promoter and a more complex pS2 promoter, elevation of cAMP did not enhance the transcription by either TOT or E2 of the reporter plasmid ERE-thymidine kinase-CAT (Fujimoto and Katzenellenbogen 1994). Thus, this phenomenon is promoter specific. Of note, cAMP and PKA catalytic subunit transfection failed to evoke transcription by the more

pure antiestrogen ICI164,384 with any of the promoter–reporter constructs tested. These findings, which document that stimulation of the PKA signaling pathway activates the agonist activity of tamoxifen-like antiestrogens, may in part explain the development of tamoxifen resistance by some ER-containing breast cancers. They also suggest that the use of antiestrogens such as ICI164,384 that fail to activate ER transcription in the presence of cAMP may prove more effective for long-term antiestrogen therapy in breast cancer.

2.5 Phosphorylation of the ER

Since our data suggested that estrogens, and agents that activate protein kinases, might influence ER transcription by altering the state of phosphorylation of the ER and/or other factors required for ER regulation of transcription, we undertook studies to examine directly the effects of these agents on ER phosphorylation. In addition, we compared the effects of the type I and type II antiestrogens on phosphorylation of the ER. E2, the antiestrogens, *trans*-hydroxy-tamoxifen and ICI164,384, as well as PKA and PKC activators enhanced overall ER phosphorylation, and in all cases, this phosphorylation appeared to be on serine residues (Le Goff et al. 1994).

Tryptic phosphopeptide patterns of wild-type and domain A/B-deleted receptors and site-directed mutagenesis of several serines involved in known protein kinase consensus sequences allowed us to identify serine 104 and/or serine 106 and serine 118, all three being part of a serine-proline motif, as major ER phosphorylation sites. Mutation of these serines to alanines so as to eliminate the possibility of their phosphorylation resulted in an approximately 40% reduction in transactivation activity in response to E_2 while mutation of only one of these serines showed an approximately 15% decrease in activation (Le Goff et al. 1994). Of note, E_2 and antiestrogen-occupied ERs showed virtually identical two-dimensional phosphopeptide patterns, suggesting similar sites of phosphorylation. In contrast, the cAMP-stimulated phosphorylation likely occurs on different phosphorylation sites as indicated by some of our mutational studies (Le Goff et al. 1994) and this aspect remains under investigation in our laboratory.

Related studies in COS-1 cells by the Chambon laboratory (Ali et al. 1993) also identified serine 118 as being a major estrogen-regulated phosphorylation site. In MCF-7 cells, the Notides laboratory has also identified S118 as a site of ER phosphorylation but has observed S167 to be the most prominent site of phosphorylation in these cells (Arnold et al. 1994). Aurrichio and coworkers (Castoria et al. 1993) have also provided strong evidence for ER phosphorylation on tyrosine 537. The roles of these phosphorylations in the activities (transcriptional and other) of the ER remains an area of great interest.

2.6 Antiestrogen Selectivity and Promoter Dependence in the cAMP-Dependent Signaling Pathway Involvement in Activation of the Transcriptional Activity of ERs Occupied by Antiestrogens

Our data provide strong evidence for the involvement of cAMP-dependent signaling pathways in the agonist actions of tamoxifen-like estrogen antagonists. The promoter specificity of the transcriptional enhancement phenomenon suggests that factors in addition to ER are probably being modulated by PKA pathway stimulation. The findings imply that changes in the cAMP content of cells, which can result in activation of the agonist activity of tamoxifen-like antiestrogens, might account, at least in part, for the resistance to antiestrogen therapy that is observed in some breast cancer patients. Of interest, MCF-7 cells transplanted into nude mice fail to grow with tamoxifen treatment initially, but some hormone-resistant cells grow out into tumors after several months of tamoxifen exposure (Gottardis and Jordan 1988; Jordan and Murphy 1990; Osborne et al. 1991). Studies have shown that this resistance to tamoxifen is, more correctly, a reflection of tamoxifen stimulation of proliferation, representing a change in the interpretation of the tamoxifen–ER complex and its agonist/antagonist balance. It is of interest that we found the pS2 gene, which is under estrogen and antiestrogen regulation in breast cancer (Brown et al. 1984), to be activated by tamoxifen in the presence of elevated cAMP. By contrast, however, antiestrogens such as ICI164,384, shown in many systems to be more complete estrogen antagonists, are not changed in their agonist/antagonist balance by increasing intracellular concentrations of cAMP. Therefore, ICI164,384-like compounds may prove to be more efficacious and less likely to result in antiestrogen-stimulated growth.

The transcriptional enhancement we have observed between PKA activators and ER occupied by tamoxifen-like antiestrogens and E_2 provides further evidence for cross-talk between the ER and signal transduction pathways regulated by cAMP that are important in ER-dependent responses.

2.7 Bidirectional Cross-Talk Between Estrogen and cAMP Signaling Pathways

For many years, steroid hormones and peptide hormones have been considered to act via distinctly different mechanisms, the former via intracellular receptors acting through the genome and the latter via membrane-localized receptors that initially affect extranuclear activities, including the generation of second messengers such as cAMP. However, there has been increasing evidence for interactions between cAMP and estrogen in enhancing the growth of the mammary gland and breast cancer cells (Sheffield and Welsch 1985; Silberstein et al. 1984) and for cAMP induction of estrogen-like uterine growth (see Aronica et al. 1994, references therein). As early as 1967, Szego and Davis (1967) demonstrated a very rapid, acute elevation of uterine cAMP by estrogen treatment of rats in vivo that was confirmed in other reports, but several subsequent studies either failed to confirm this observation or reported only minimal effects that were considered to represent indirect effects of estrogen on cAMP mediated by estrogen-induced release of uterine epinephrine (see Aronica et al. 1994, references therein). Recently, cAMP and other protein kinase activators have been documented to synergize with steroid hormone-occupied receptors, leading to enhanced receptor-mediated transcription (Aronica and Katzenellenbogen 1993; Beck et al. 1993; Cho and Katzenellenbogen 1993; Fujimoto and Katzenellenbogen 1994; Groul and Altschmeid 1993; Sartorius et al. 1993), possibly by a mechanism involving phosphorylation of the receptor or associated transcription factors (Ali et al. 1993; Aronica and Katzenellenbogen 1993; Le Goff et al. 1994; Montminy et al. 1986).

Our recent studies have shown that estrogen activates adenylate cyclase, markedly increasing the concentration of cAMP in estrogen-re-

sponsive breast cancer and uterine cells in culture and in the intact uterus of rats treated with estrogen in vivo, in a manner that does not require new RNA or protein synthesis (Aronica et al. 1994). The intracellular concentrations of cAMP achieved by low, physiological levels of estrogen are substantial and sufficient to stimulate cAMP response element (CRE)-mediated gene transcription. Therefore, this nongenomic action of the steroid hormone estrogen involves the production of an important second messenger and the resultant activation of second messenger-stimulated genes. These findings document a two-way directionality in the cross-talk between steroid hormone- and cAMP-signaling pathways.

Although the hormonal specificity in the stimulation of cAMP is consistent with it being mediated by a high-affinity, ER-like binder, the nature of the potential membrane binder remains to be determined. Likewise, the mechanism by which estrogen enhances intracellular cAMP levels remains to be further examined. Several other publications have indicated an important role for sex hormone-binding globulin in the actions of sex steroids in enhancing intracellular cAMP (Fissore et al. 1994, Fortunati et al. 1993). The role of serum factors, including sex hormone-binding globulin, remains an important aspect for future analyses. These current observations imply a possibly broad involvement of steroid hormone action on cyclic nucleotide and second-messenger systems. The increasing evidence for rapid, membrane effects of estrogens and progestins and for vitamin D in a variety of target cells suggests that this aspect of steroid hormone action deserves further investigation (Ke and Ramirez 1987, 1990; Kim and Ramirez 1986; Lieberherr et al. 1993; Pappas et al. 1995).

Thus, data from this laboratory and others provide increasing evidence for extensive two-way cross-talk between estrogen and cAMP signaling pathways: in one way, cAMP is able to enhance the transcription of estrogen-regulated genes containing EREs; in the second way, estrogens appear able to act via the cAMP system to potentially regulate cAMP-mediated gene transcription. Further analyses of the underlying mechanisms in a variety of target cells should provide further insights in understanding the biology and regulation of estrogen-responsive cells.

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3 Analysis of Genetically Altered Mice Without Glucocorticoid Receptor

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3.1 Introduction

Steroid hormones regulate a number of developmental and physiological processes in vertebrates by controlling the transcriptional activity of specific genes (Beato 1989, Tsai and O'Mally 1994). The ability of target cells to respond is attributed to the presence of specific receptors which mediate the action of the hormone within the cell. The receptors are localized within the nucleus in association with other proteins, which in absence of the hormone keep the receptor in an inactive state. After binding of the hormone, the hormone–receptor complex, as a dimer, binds to specific DNA sequences. The various functions of the receptor – DNA binding, ligand binding, transcriptional activation – have been assigned to separate domains of the receptor. The unliganded receptor is maintained in a nonfunctional form by oligomerization with

SYMPOSIUM 6: STEROID HORMONES: BREAST AND PROSTATE CANCER

Monday, April 22, 8:00–11:00 a.m., Hall A

Monday, April 22, 8:05–8:35 a.m., Hall A

Estrogen receptor bioactivities and interactions with signal transduction pathways in breast cancer cells. Benita S. Katzenellenbogen, *Departments of Molecular & Integrative Physiology, Cell & Structural Biology, University of Illinois, College of Medicine, Urbana, IL 61801.*

We are interested in understanding how estrogens, antiestrogens and growth factors regulate the proliferation and properties of human breast cancer cells. Fascinating interconnections exist among the different signaling pathways stimulated by the multiple factors impinging on breast cancer cells. Since each signaling pathway involves an effector molecule interacting with a receptor to cause a response, attention has been directed to understanding the nature of the effector ligand-receptor interaction, the factors that regulate the levels of these receptors by controlling their synthesis and degradation, and the factors that modulate the activity of the ligand-receptor complexes. The actions of estrogens in stimulating gene transcription and ultimately cell proliferation are modulated by progestins and antagonized by antiestrogens. In an effort to understand how antiestrogens act, we have used affinity labeling and site directed and random chemical mutagenesis to investigate how estrogen receptor (ER) discriminates between agonists and antagonists, and how these ligands influence subsequent chromatin/gene interactions of the receptor (1). We have found that the level and activity of estrogen receptors and progesterone receptors are influenced by hormone, antihormone, growth factors and activators of protein kinases. The observation that these agents influence the efficacy of the ER in stimulating gene transcription and that protein kinase inhibitors and antiestrogens suppress the stimulation of ER-mediated gene activation suggested the likely involvement of phosphorylation pathways. Direct phosphorylation studies document that many of these agents do alter the magnitude of ER phosphorylation, and specific sites of phosphorylation have been found to alter transcriptional activity and other properties of the receptor (2,3). The response to estrogen involves alteration in the production of growth factors, growth factor receptors and protooncogenes believed to be involved in the growth response, as well as stimulation of progesterone receptor production which increases the cells' sensitivity to progestin, and production of intracellular and secreted proteins including plasminogen activators that may play a role in increasing the metastatic potential of the cells.

Antiestrogens, acting via the estrogen receptor, evoke conformational changes in the ER and inhibit the effects of estrogens. Although the binding of estrogens and antiestrogens is mutually competitive, studies with ER mutants indicate that some of the contact sites of estrogens and antiestrogens are likely different. Some mutations in the hormone binding domain of the ER and deletions of carboxyl-terminal regions result in ligand discrimination mutants, i.e. receptors that are differentially altered in their ability to bind and mediate the actions of estrogens vs. antiestrogens (1,4). Mutations in the activation function-2 region result in ERs which are transcriptionally inactive with estradiol and have potent dominant negative inhibitory activity, being able to suppress the actions of wild type ER at low molar ratios (5). We have examined the role of specific ER functions and domains in this transcriptional repression and find that competition for estrogen response element binding, formation of inactive heterodimers and specific transcriptional silencing can all contribute to the dominant negative phenotype, and that these receptors suppress the activity of the wild type ER by acting at multiple steps in the ER-response pathway (6).

Studies in a variety of cell lines and with different promoters indicate marked cell context- and promoter-dependence in the actions of antiestrogens and variant ERs. In several cell systems, estrogens and protein kinase activators such as cAMP synergize to enhance the transcriptional activity of the ER in a promoter-specific manner (7). In addition, cAMP changes the agonist/antagonist balance of tamoxifen-like antiestrogens, increasing their agonistic activity and reducing their efficacy in reversing estrogen actions (8). Estrogens, as well as protein kinase activators such as cAMP and some growth factors increase phosphorylation of the ER and/or proteins involved in the ER-specific response pathway. These changes in phosphorylation alter the biological effectiveness of the ER. Receptor mutants are being utilized to map sites on the ER important in this transcriptional synergism/enhancement. Our observations suggest that changes in cellular phosphorylation state will be important in determining the biological activity of the ER and the effectiveness of antiestrogens as estrogen antagonists.

We have developed several model cell systems for studying the changes in breast cancer cells that accompany the progression from hormone-dependence to hormone-autonomy and some changes associated with the development of antiestrogen resistance and, hence, failure to respond to antiestrogen therapy. These estrogen growth-autonomous and tamoxifen-resistant breast cancer cell lines show changes in their production of, and responsiveness to, TGFα and TGFβ (9). These alterations may accompany the conversion of the cells to an antiestrogen growth-resistant phenotype. Multiple interactions among different cellular signal transduction systems are involved in the regulation of breast cancer cell proliferation and gene expression by estrogens and antiestrogens.

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Different Regions in Activation Function-1 of the Human Estrogen Receptor Required for Antiestrogen- and Estradiol-dependent Transcription Activation*

(Received for publication, April 23, 1996, and in revised form, June 5, 1996)

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The human estrogen receptor (ER) is a ligand-inducible transcription factor that contains two transcriptional activation functions, one located in the NH_2-terminal region of the protein (AF-1) and the second in the COOH-terminal region (AF-2). Antiestrogens, such as *trans*-hydroxytamoxifen (TOT), have partial agonistic activity in certain cell types, and studies have implied that this agonism is AF-1-dependent. We have made progressive NH_2-terminal and other segment deletions and ligations in the A/B domain, and studied the transcriptional activity of these mutant ERs in ER-negative MDA-MB-231 human breast cancer and HEC-1 human endometrial cancer cells. Using several estrogens and several partial agonist/antagonist antiestrogens, we find that estrogens and antiestrogens require different regions of AF-1 for transcriptional activation. Deletion of the first 40 amino acids has no effect on receptor activity. Antiestrogen agonism is lost upon deletion to amino acid 87, while estrogen agonism is not lost until deletions progress to amino acid 109. Antiestrogen agonism has been further defined to require amino acids 41–64, as deletion of only these amino acids results in an ER that exhibits 100% activity with E_2, but no longer shows an agonist response to TOT. With A/B-modified receptors in which antiestrogens lose their agonistic activity, the antiestrogens then function as pure estrogen antagonists. Our studies show that in these cellular contexts, hormone-dependent transcription utilizes a range of the amino acid sequence within the A/B domain. Furthermore, the agonist/antagonist balance and activity of antiestrogens such as TOT are determined by specific sequences within the A/B domain and thus may be influenced by differences in levels of specific factors that interact with these regions of the ER.

The estrogen receptor (ER)[1] is a ligand-inducible transcription factor that regulates gene expression through interaction with cis-acting DNA elements called estrogen response elements (EREs) (for reviews, see Refs. 1–5). Like other steroid hormone receptors, the ER contains specific domains responsible for functions leading to transcription of target genes, such as ligand binding, DNA binding, and transactivation (6–8). The ER contains two distinct, non-acidic activation functions, one activation function at the NH_2 terminus (AF-1) and a second, hormone-dependent activation function at the COOH terminus (AF-2), in the hormone binding domain (8–12). AF-2 is highly conserved among species and other nuclear hormone receptors (1, 12, 13), whereas the A/B domain at the amino terminus of the ER, which includes AF-1, is less well conserved among different species and other nuclear receptors (1, 13, 14). The activity of each activation function of ER is cell- and gene promoter-dependent. AF-1 can exhibit transcriptional activity in the absence of AF-2 (8) in some cell contents but, in most cell and promoter contexts, both AF-1 and AF-2 function in a synergistic manner and are required for full receptor activity (6, 8, 15–22).

Transactivation of estrogen-responsive genes by ER can be antagonized by antiestrogens such as *trans*-hydroxytamoxifen (TOT) and ICI 164,384 (18, 19). One mechanism by which these antiestrogens inhibit ER action is by competition with estradiol (E_2) for binding to the ER. Although antiestrogen-occupied ER binds estrogen response DNA elements in cells (23, 24), it is thought that antiestrogens promote a conformational change which is different from that induced by E_2 (24, 25). Some antiestrogens, like TOT, have partial agonistic activity in certain cells, such as chicken embryo fibroblasts (CEF) and MDA-231 human breast cancer cells (18, 26). The cell and promoter dependence of TOT agonism has been attributed to the cell and promoter specificity of AF-1 activity (15–18). Previous studies using chimeric receptors have shown that TOT is unable to induce AF-2 activity, but that TOT can be a strong agonist in cellular and promoter contexts where AF-1 is an efficient transcriptional activator (11, 18, 21).

We have investigated the A/B domain of the ER and its role in the transcriptional activity of ER elicited by estrogens and some antiestrogens, and we find that different regions within this domain are required for transcriptional stimulation by estrogen *versus* antiestrogen. In the studies presented, we demonstrate that a specific 24-amino acid region of AF-1 of the human ER is necessary for agonism by TOT and other partial agonist/antagonist antiestrogens, but is not required for E_2-dependent transactivation. As a consequence, the activity of estradiol and the estrogen agonist/antagonist character of TOT depended markedly, but not always concordantly, on the se-

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[1] The abbreviations used are: ER, estrogen receptor; hER, human estrogen receptor; ERE, estrogen response element; E_2, 17β-estradiol; TOT, *trans*-hydroxytamoxifen; AF, activation function; CAT, chloramphenicol acetyltransferase; HEC-1, human endometrial cancer cells; CEF, chicken embryo fibroblast cells; CMV, cytomegalovirus, BF, 2-phenylbenzofuran; BT, 2-phenylbenzothiophene; PCR, polymerase chain reaction.

quences present within the A/B domain in the ER. Our studies show that in the context of the full-length ER, hormone-dependent transcription utilizes a broad range of sequences within the A/B domain and suggest that differences in the agonist/antagonist character of antiestrogens observed in different cells could be due to altered levels of specific factors that interact with these regions.

MATERIALS AND METHODS

Chemicals and Materials—Cell culture media were purchased from Life Technologies, Inc. Calf serum was from Hyclone Laboratories (Logan, UT) and fetal calf serum was from Sigma. [^{14}C]Chloramphenicol (50–60 Ci/mmol) was from DuPont NEN. The antiestrogens TOT and ICI 164,384 were kindly provided by Dr. Alan Wakeling, Zeneca Pharmaceuticals, Macclesfield, United Kingdom. The antiestrogens 2-phenylbenzofuran (BF) and 2-phenylbenzothiophene (BT) were generously provided by Dr. E. von Angerer, University of Regensburg, Germany.

Plasmid Constructions—The ER expression vectors, all containing human ER (hER), are derivatives of pCMV5-hER (27). NH_2-terminal deletion mutants N21 and E41 were constructed by replacement of the pCMV5-hER *Sst*II fragment with a PCR-generated fragment containing a new start codon and an *Sst*II site at amino acids 21 and 41, respectively. NH_2-terminal deletion mutants A87 and M109 were constructed by replacement of the pCMV5-hER *Sst*II/XmaIII fragment with a PCR-generated fragment containing an *Sst*II site at amino acids 87 and 109, respectively. Estrogen receptor deleted of amino acids 41–64 (Δ41–64) was constructed by replacing the *Sst*II fragment of pCMV5-hER (containing residues 1–64) with a PCR-generated fragment containing residues 1–40 with an *Sst*II site after amino acid 40. Δ87–108 was constructed by inserting a PCR-generated fragment containing an *Sst*II site at amino acid 87 into the *Sst*II site of M109 and insertion of the *Xma*III fragment from this construct to replace the *Xma*III fragment of pCMV5-hER. 41–66-CDEF was constructed by replacing the *Xma*III fragment of E41 with a PCR-generated fragment containing an *Xma*III site at amino acid 180. 41–87-CDEF was constructed by replacing the *Xma*III fragment from pCMV5-hER with two PCR-generated fragments, amino acids 41–87 and amino acids 180–311 containing *Bgl*II sites at amino acids 88 and 179. 41–109-CDEF was constructed in a similar manner to 41–87-CDEF with a PCR-generated fragment, amino acids 41–109, containing a *Bgl*II site at amino acid 110. ΔAB ER was constructed as described previously (28). The sequences of all ER mutants utilized were confirmed by dideoxy sequencing methods to assure accuracy. The $(ERE)_3$-pS2-chloramphenicol acetyltransferase (CAT) reporter was constructed as described previously (27). The plasmid pCMVβ, which contains the β-galactosidase gene, was used as an internal control for transfection. The plasmid pTZ19R, used as carrier DNA, was provided by Dr. Byron Kemper of the University of Illinois.

Cell Culture and Transient Transfections—MDA-MB-231 human breast cancer cells were maintained in Leibovitz's L-15 Medium with 10 mM HEPES, 5% calf serum, 100 units of penicillin/ml (Life Technologies, Inc.), 100 μg of streptomycin/ml (Life Technologies, Inc.), 25 μg of gentamycin/ml, 6 ng of bovine insulin/ml, 3.75 ng of hydrocortisone/ml, and 16 μg of glutathione/ml. Human endometrial cancer (HEC-1) cells were maintained in minimum essential medium plus phenol red supplemented with 5% calf serum and 5% fetal calf serum, 100 units of penicillin/ml (Life Technologies, Inc.), and 100 μg of streptomycin/ml (Life Technologies, Inc.). MDA-231 cells or HEC-1 cells were grown in minimum essential medium plus phenol red supplemented with 5% charcoal/dextran-treated calf serum for 2 days prior to transfection. Cells were plated at a density of 3×10^6 cells/100-mm dish in phenol red-free Improved minimal essential medium and 5% charcoal/dextran-treated calf serum and were given fresh medium 24 h before transfection. All cells for transfection were maintained at 37 °C in a humidified CO_2 atmosphere. Cells were transiently transfected by the $CaPO_4$ coprecipitation method (29). One ml of precipitate contained 0.8 μg of pCMVβ as internal control, 6 μg of an ERE-containing reporter plasmid $(ERE)_3$-pS2-CAT, 100 ng of ER expression vector, and pTZ19R carrier DNA to a total of 15 μg of DNA. Cells remained in contact with the precipitate for 4 h and were then subjected to a 2.5-min glycerol shock (20% in transfection medium). Cells were rinsed with Hanks' balanced salt solution and given fresh medium with hormone treatment as indicated.

Promoter Interference Assays—MDA-MB-231 cells were transiently transfected with 2 μg of CMV-$(ERE)_2$-CAT reporter plasmid (23), 0.8 μg of pCMVβ, 12.2 μg of pTZ19R, and 100 ng of ER expression vector/100-mm dish of cells. Cells were treated as described previously for transient transfection, and CAT assays were performed on cell extracts.

Immunoblot Assays—COS-1 cells were transfected in 100-mm dishes with 10 μg of expression vector for wild type ER or ER derivatives and 5 μg of pTZ19R carrier plasmid. Whole cell extracts were collected by centrifugation and fractionated on a polyacrylamide gel. Proteins were transferred to nitrocellulose and immunoblots were performed using ER monoclonal antibody H222 as described previously (30).

RESULTS

Different Regions in the A/B Domain Are Important for Estradiol- and trans-Hydroxytamoxifen-dependent Transcriptional Activity—Our studies were aimed at identifying regions within the A/B domain that are responsible for E_2-dependent transcription and for antiestrogen agonism. We have generated ER derivatives that contain increasing NH_2-terminal deletions or other deletional changes in the A/B domain. Fig. 1 shows the structure of the ER derivatives used in this study and the relative expression levels of the receptors observed in cells. Western immunoblot analysis showed that receptors of the predicted sizes were being produced in the cells and that all of the A/B domain altered receptors (Fig. 1*B*) were expressed at levels very similar to that of the wild type ER.

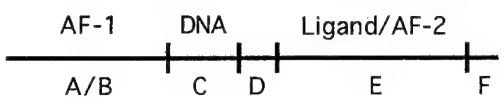
These ER mutants were then analyzed for their ability to transactivate an ERE-containing pS2 promoter-reporter gene in ER-negative MDA-231 human breast cancer cells. Wild type ER or receptors with deletions of amino acids 1–20 (N21), 1–40 (E41), 1–86 (A87), 1–108 (M109), or 1–179(ΔAB) were transiently transfected into MDA-231 cells, and transcriptional activity was measured in response to increasing concentrations of E_2. ER mutants N21, E41, and A87 showed dose-response curves for transcriptional activity virtually identical to that observed with wild type ER (Fig. 2*A*). In contrast, deletion of the first 108 amino acids resulted in receptors that showed a great loss of activity; M109 receptors showed only about 20% of wild type ER transcriptional activity at 10^{-8} M E_2, suggesting that residues between amino acid 87 and 108 are important for estradiol-stimulated activity. Deletion of the complete A/B domain (amino acids 1–179) gave a receptor that showed no activity in this cell system.

Similar studies were conducted using the NH_2-terminal deletion mutants to examine transcriptional response to the triphenylethylene compound *trans*-hydroxytamoxifen, TOT (Fig. 2*B*). MDA-231 cells were again used in these studies, since with wild type ER, TOT behaves as a relatively strong agonist. TOT (10^{-7} M) stimulates transcriptional activity to approximately 30% the level evoked by maximal (10^{-8} M) E_2 stimulation. Compared with the wild type ER, deletion of amino acids 1–20 or 1–40 had no effect on either the E_2 response or TOT agonism. However, deletion of amino acids 1–86, which had no effect on E_2-induced activity, abolished TOT agonism completely (Fig. 2B). The further deleted mutant, M109, which was transcriptionally impaired in response to E_2 treatment, did not exhibit any measurable response to TOT. The loss of TOT agonism observed selectively with the A87 mutant suggested that sequences between 41 and 87 may be important contributors to TOT agonism, but are not essential for the response to E_2.

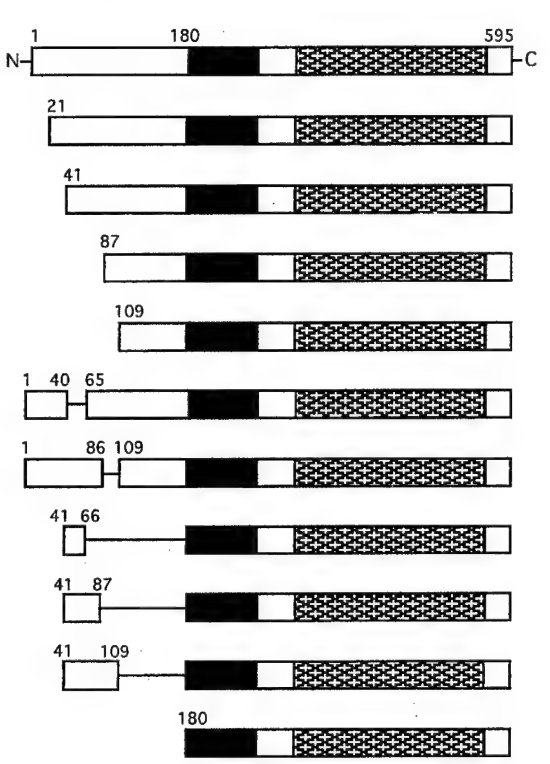
Deletion mutant Δ41–64, which lacks only amino acids 41–64, was constructed and tested for its transactivation ability in response to E_2 and TOT. Δ41–64 retained 100% of wild type E_2-dependent activity (Fig. 2*C*) yet displayed no measurable response to TOT (Fig. 2*D*). These results are consistent with the loss of TOT response with the A87 mutant as they implicate residues 41–64 as a major contributor to TOT agonism but not to E_2 response.

A/B Deletion Mutants Exhibit Differential Response to Other Estrogens and Antiestrogens—Further examination of the ligand-dependent transcriptional activity of these mutants revealed

A



	E_2	TOT
1) WT ER	100%	100%
2) N21	102 ± 5	92 ± 12
3) E41	116 ± 20	98 ± 13
4) A87	105 ± 28	5 ± 1
5) M109	21 ± 3	3 ± 0.4
6) Δ41-64	104 ± 13	3 ± 0.7
7) Δ87-108	72 ± 3	49 ± 5
8) 41-66-CDEF	37 ± 3	7 ± 2
9) 41-87-CDEF	32 ± 6	7 ± 1
10) 41-109-CDEF	82 ± 15	83 ± 14
11) ΔAB	0.5 ± 0.2	0.8 ± 0.2



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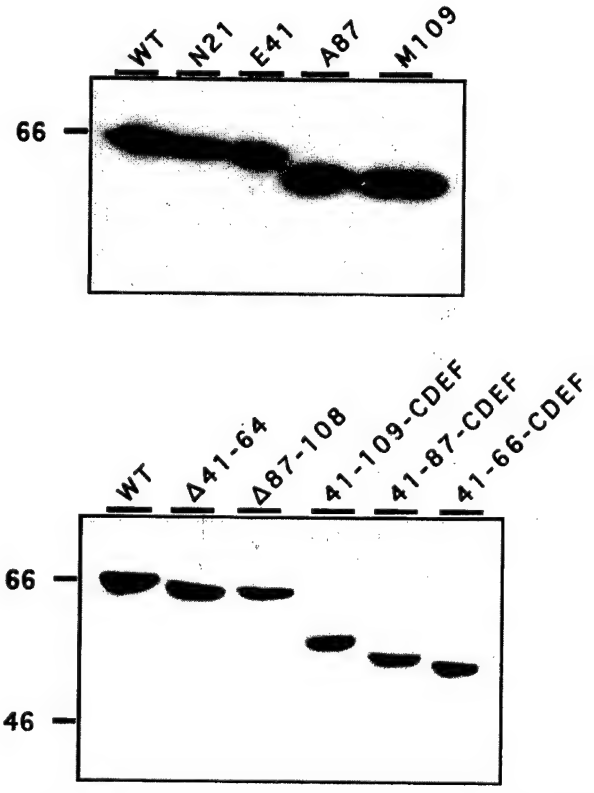

FIG. 1. **Structure and expression of ER derivatives.** *A*, the functional domains (A/B, C, D, E, F) and activation functions (AF-1 and AF-2) of ER are shown at the *top* along with schematics for the A/B domain mutants studied in this report. The values to the *right* of the receptor

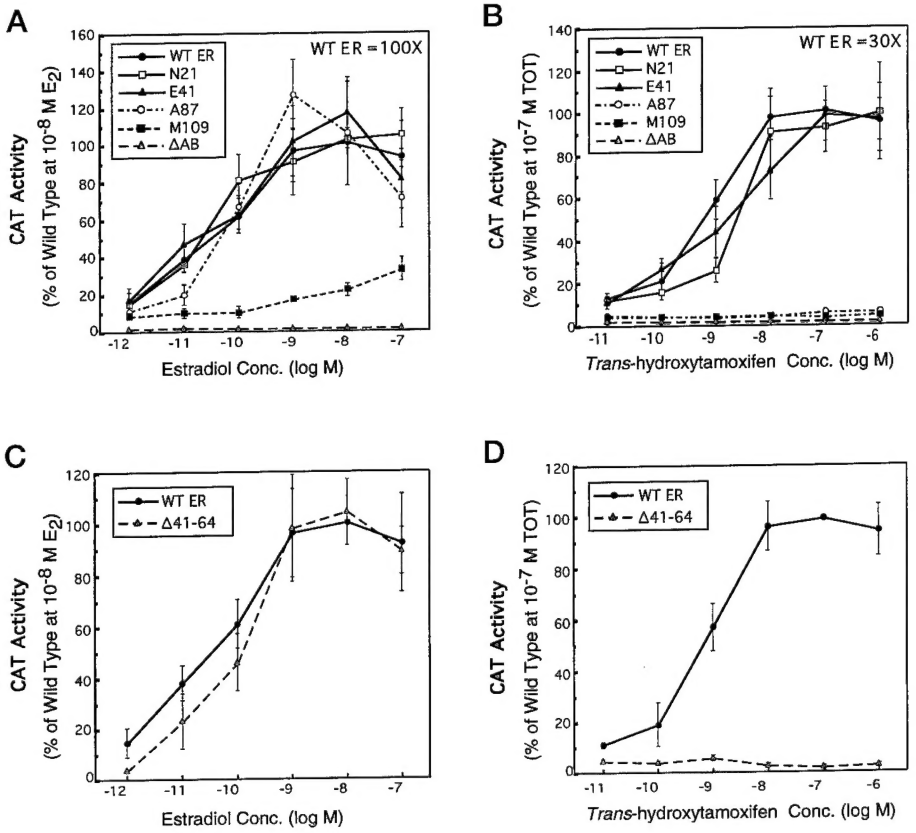

FIG. 2. **Transcriptional activation by wild type ER and A/B domain deletion ER mutants.** ER-negative MDA-231 cells were transfected with expression vector for wild type or mutant ER and a $(ERE)_3$-pS2-CAT reporter gene. Cells were treated with increasing concentrations of E_2 (*A* and *C*) or TOT (*B* and *D*) for 24 h. CAT activity was normalized for β-galactosidase activity from an internal control plasmid. Values represent the mean ± S.E. for three or more determinations and are expressed as a percentage of wild type ER response with 10^{-8} M E_2 or 10^{-7} M TOT. For some values, *error bars* are too small to be visible. Wild type ER showed a ~100-fold and 30-fold induction of CAT activity in response to 10^{-8} M E_2 or 10^{-7} M TOT, respectively.

that another full estrogen, the resorcylic lactone P1496 (31), showed a pattern of activity identical to that observed with E_2. Like E_2, transcriptional response to P1496 was fully retained in N21, E41, and A87 receptors, but was impaired with the deletion of the first 108 residues (Fig. 3*A*). Similar results to those seen with TOT were observed with the antiestrogen compounds BF and BT (32). Like TOT, these heterocycle-based antiestrogens were significant agonists, evoking transcriptional activity that was similar in magnitude to that obtained with TOT (~30% of E_2 stimulation). As seen in Fig. 3*A*, antiestrogen stimulation of CAT activity was lost with the mutants A87 and Δ41–64 for the three antiestrogen compounds (TOT, BF, and BT), while estrogen (E_2 and P1496) stimulation of transcriptional activity was still maintained maximally in these two constructs. No stimulation of wild type ER or any ER mutants was seen with the pure antiestrogen ICI 164,384 (data not shown).

These A/B domain mutants were also tested in a different cell background utilizing an ER-negative human endometrial cancer cell line (HEC-1 cells). In these cells, wild type ER also responds to TOT as an agonist, showing about 30–40% of wild type E_2 response (Fig. 3*B*). Similar results to those seen previously in MDA-231 breast cancer cells were observed with the A/B domain deletion mutants in these endometrial cancer cells; both A87 and Δ41–64 receptors retained full wild type transcriptional activity in response to E_2 but did not exhibit any response to TOT. These results demonstrate again that a region between amino acids 40 and 65 is critical for TOT agonism yet is not required for E_2-dependent transcription.

Specific Regions in the A/B Domain Are Required to Support TOT Agonism—Since TOT was not a full estrogen agonist in these assays, and is known to show mixed estrogen agonist and antagonist activity in many cells (15–18), we also examined the antagonist activity of TOT and how this was impacted by changes in the A/B domain of ER (Fig. 4). TOT agonism was apparent in wild type ER, N21, and E41 receptors and, in these three receptors, TOT (at a 10-fold excess concentration relative to that of E_2) was also able to suppress E_2-stimulated activity to that of its own inherent level of agonism (*i.e.* approximately 30% of the E_2-stimulated level). Thus, with these receptors, this compound showed partial agonist and partial antagonist activity. Of interest, in the A87, M109, and Δ41–64 receptors where TOT showed no agonistic activity, TOT behaved as a pure antiestrogen and was now a complete antagonist of the E_2 stimulation. Thus, the agonist/antagonist character of the antiestrogen TOT differed with the nature of the ER A/B domain.

Deletions in the A/B Domain Do Not Affect Receptor Level or DNA Binding—Since certain A/B deletion mutants exhibited a differential response to estrogens and antiestrogens, the levels of these receptors and the DNA binding abilities of these mutant ERs were determined following exposure to E_2 or TOT in order to determine whether differences in response to these two ligands might be attributable to ligand-induced alteration in receptor stability or DNA binding ability. As seen in Fig. 5*A*, levels of wild type ER, Δ41–64 ER and A87 ER were similar following cell treatment with E_2 or TOT. Thus, differential turnover of these receptor proteins in response to TOT *versus* E_2 is not likely to explain the very different transcriptional response of these receptors to these two ligands.

DNA binding studies were conducted with several of the mutants by use of a promoter interference assay, in order to assess whether differences in DNA binding of the TOT-ER

schematics indicate the transcriptional activity of the receptors in response to 10^{-8} M E_2 or 10^{-7} M TOT and summarize data that are derived from dose response experiments detailed later in this paper. *B*, the expression of wild type and mutant estrogen receptors from cytomegalovirus promoter-containing expression vectors was determined following transfection into ER-negative COS-1 cells. Equal amounts of protein were used and immunoblotting was done with the anti-ER monoclonal antibody H222.

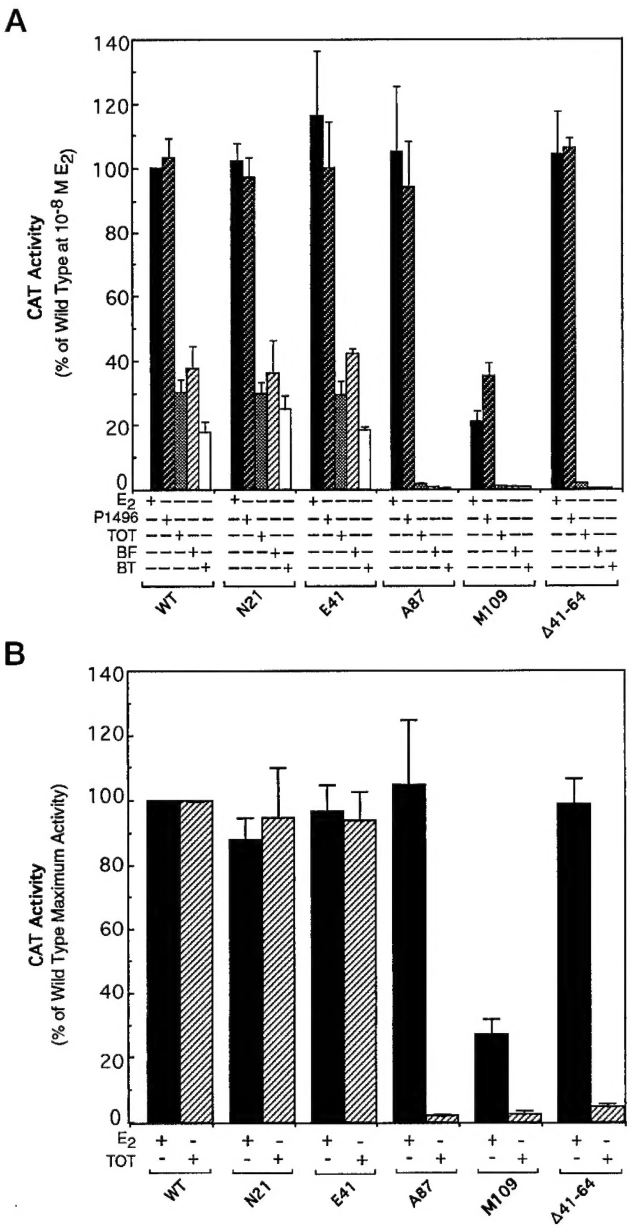

FIG. 3. **Transcriptional activation by wild type ER and A/B domain deletion ER mutants in response to two estrogens and three antiestrogens.** *A*, MDA-231 breast cancer cells were transfected with ER expression vectors and a $(ERE)_3$-pS2-CAT reporter gene. Cells were treated for 24 h with either 10^{-8} M E_2, 10^{-7} M P1496, 10^{-7} M TOT, 10^{-7} M BF, or 10^{-7} M BT as indicated. *B*, ER-negative HEC-1 human endometrial cancer cells were transfected with ER expression vectors and a $(ERE)_3$-pS2-CAT reporter gene and treated with either 10^{-8} M E_2 or 10^{-7} M TOT. CAT activity was determined as described in the legend to Fig. 2. Values are the mean ± S.E. for three or more determinations from separate experiments. Some error bars are too small to be visible.

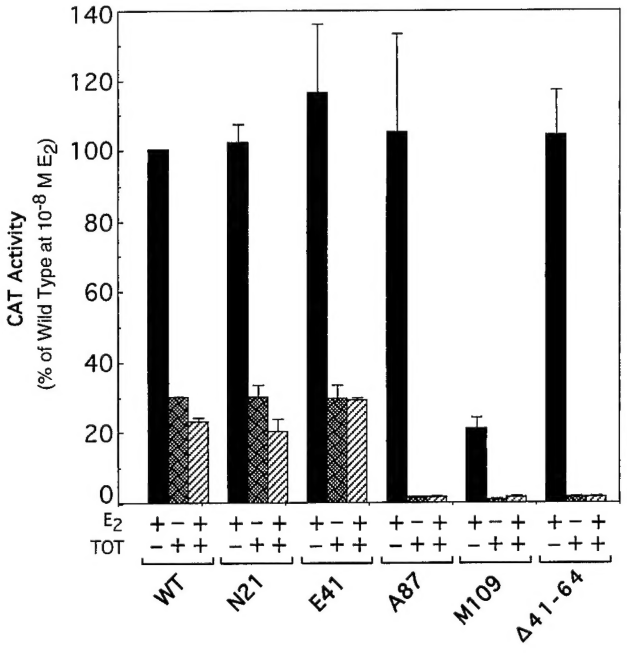

FIG. 4. **The antiestrogen TOT is an estrogen agonist and antagonist, with its agonist/antagonist balance dependent on the particular ER protein.** ER-negative MDA-231 cells were transfected with expression vector for wild type or A/B domain ER mutants and a $(ERE)_3$-pS2-CAT reporter gene. Cells were treated for 24 h with 10^{-8} M E_2 or 10^{-7} M TOT alone or in combination (10^{-8} M E_2 and 10^{-7} M TOT). CAT activity was analyzed as described in the legend to Fig. 2. Values are the mean ± S.E. for three or more determinations from separate experiments. Some error bars are too small to be visible.

versus E_2·ER complexes might explain their different transcriptional efficacy (Fig. 5*B*). This promoter interference assay measures the ability of ER to bind to ERE DNA in intact cells (23). Binding of ER to the ERE is assayed by assessing the ability of ERE-bound ER to block transcription from the constitutively active cytomegalovirus (CMV) promoter, with the repression of CAT activity being a measure of the binding of ER to the ERE-containing promoter. A87, which responds to E_2 but not to TOT, and M109, which is impaired in both E_2- and TOT-dependent activity, were both able to bind to the EREs and to interfere with promoter activity to the same extent as the wild type ER (Fig. 5*B*). Therefore, differences in E_2- and TOT-dependent transactivation exhibited by these ER derivatives do not appear to be caused by differences in receptor protein level or by differential DNA binding.

Residues 41–109 Encompass Sequences Important for Both Estradiol- and TOT-dependent Transcription—Additional analysis of the A/B region was made to further characterize sequences important for E_2- and TOT-dependent transcription. Since transcriptional response to E_2 was almost completely lost in going from the A87 to the M109 ER, we wished to directly assess the importance of amino acids 87–108 in E_2-dependent activity. To do so, we tested an ER mutant lacking only amino acids 87–108 (Δ87–108). Full dose-response studies employing 10^{-12} to 10^{-7} M E_2 and 10^{-11} to 10^{-6} M TOT were conducted for this mutant and all other mutants described below, as done for the mutant ERs shown in Fig. 2. The dose-response curves are not shown, but the findings at 10^{-8} M E_2 and 10^{-7} M TOT are summarized in Fig. 1*A*. Deletion of residues 87–108 resulted in only a ~30% decrease in E_2-stimulated transcriptional activity (Fig. 1*A*, entry *7*). From these results, it appears that E_2-dependent transcription is supported by sequences outside of the 87–108 region of the A/B domain, as deletion of only these amino acids is not sufficient to reduce the transcriptional activity to the level observed with M109.

Further analysis of the A/B region was made using segment ligated mutants (Fig. 1*A*, entries *8–10*). To examine the region between residues 40 and 65, which were required for TOT agonism, we constructed a segment ligated ER derivative, 41–66-CDEF, containing only amino acids 41–66 of the A/B domain linked directly to the intact ER domains C through F and assayed this receptor for its ability to transactivate an ERE-containing reporter gene in the presence of E_2 or TOT. This mutant was surprising in its ability to activate the reporter gene to approximately 40% of the wild type ER in response to E_2 (Fig. 1*A*, entry *8*), even though deletion of amino acids 41–64 resulted in no change in E_2-stimulated activity. The ER mutant

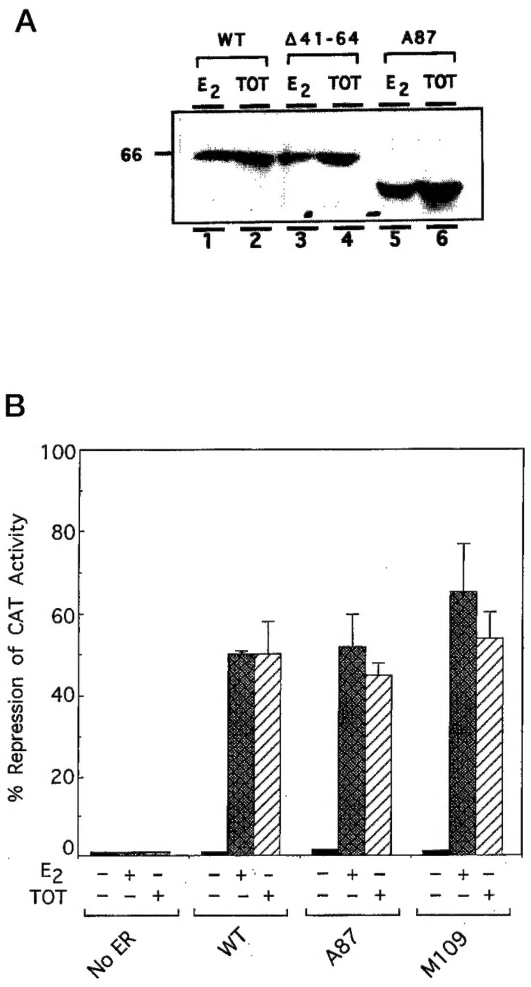

FIG. 5. **Protein levels and DNA-binding abilities of wild type ER and ER mutants treated with estrogen or antiestrogen.** *A*, levels of wild type ER and ER mutants were examined following transfection and treatment of COS-1 cells with either 10^{-8} M E_2 or 10^{-7} M TOT for 24 h. Immunoblotting was done with the anti-ER monoclonal antibody H222. *B*, MDA-231 cells were transfected with the constitutively active CMV-$(ERE)_2$-CAT promoter interference plasmid and wild type ER or mutant ERs. Cells were treated with control vehicle, 10^{-8} M E_2, or 10^{-7} M TOT, and CAT activity was analyzed as described in the legend to Fig. 2. Values are the mean ± S.E. for three or more determinations from separate experiments. For some values, error bars are too small to be visible.

41–66-CDEF, however, exhibited no measurable response to TOT. This suggests that residues 41–64 are necessary for TOT agonism, but that they alone are not sufficient for TOT-directed transcription. Extension of the A/B domain toward the COOH terminus (Fig. 1*A*, entry *9*) to include amino acids 41–87 (41–87-CDEF) did not result in any increase in E_2- or TOT-dependent transcription compared with 41–66-CDEF. However, extension to amino acid 109 (41–109-CDEF) did result in a 2-fold increase in E_2-dependent transcriptional activity compared with 41–66-CDEF and a dramatic increase in TOT agonism such that the activity measured was approximately 80% of wild type ER activity for both E_2 and TOT (Fig. 1*A*, entry *10*). This indicates that the region encompassing residues 41–109 contains almost all of the A/B domain sequence needed both for E_2 and TOT stimulatory activity.

Interestingly, the transcriptional activity of 80% observed with 41–109-CDEF is in agreement with the observation that only 20% of wild type E_2-stimulated activity is retained upon deletion of the first 108 residues. These results suggest that residues 87–108 play a significant role in E_2-stimulated transcriptional activity but are supported by other sequences in the A/B domain. This is highlighted by the Δ87–108 mutant (Fig. 1*A*, entry *7*), which lacks residues 87–108 in the A/B domain. This mutant is only weakly impaired in response to E_2 and TOT compared with wild type ER, consistent with residues 41–109 being important for full AF-1 function. Together, these results demonstrate that E_2- and TOT-dependent transcription utilizes other flanking sequences beyond amino acids 87–108 within the A/B domain to achieve full receptor activity. These required regions could serve as a portion of the activation function or could serve a structural purpose, perhaps maintaining proper three-dimensional structure of the receptor protein.

DISCUSSION

The human estrogen receptor contains two transcriptional activation functions, AF-1 located in the A/B domain and AF-2 in the hormone-binding domain. Both transcriptional activation functions act in a promoter- and cell type-dependent manner. The amino acid sequences of these activation functions are not similar to other known activation sequences, so elucidation of their precise mechanism of action is of interest. Our studies have defined AF-1 regions within the A/B domain of ER that support the transcriptional response to estrogens (E_2, P1496) and those that support the transcriptional response to several antiestrogens. While considerable overlap in the transcription-supporting regions is observed for both categories of ligands, we found that there are some distinct sequence requirements.

There are limitations in the applications of mutational methods to precisely define regions of the A/B domain that support the transcriptional agonism of these different ligands, as these activities appear to be distributed over more than one discrete segment. To address these issues we have, in fact, made three different types of alterations in the A/B domain, namely progressive NH_2-terminal deletions, segmental deletions, and segmental ligations. In many cases, we obtained consistent results regarding the transcription-supporting role of a particular region of the A/B domain by making the different types of mutations; however, we did not always get identical results using all three approaches.

When making progressive NH_2-terminal deletions, TOT agonism is lost when the A/B domain is truncated from E41 to A87, whereas the effect of E_2 is reduced only upon further deletion to M109. Therefore, TOT agonism appears to require a region between residues 41–86, whereas E_2 induction requires the 87–108 sequence. Segmental deletion of residues 41–64 does, in fact, eliminate TOT agonism without affecting E_2 induction. However, the 87–108 segmental deletion, which has a limited effect on TOT agonism, causes only a modest reduction in E_2 induced transcription. Thus, whereas the region 87–108 appears to be critical to the E_2 effect in the absence of residues 1–86 (*i.e.* by progressive NH_2-terminal deletion), it appears that much of the E_2 effect can be supported by the 1–86 segment (perhaps together with the 109–180 segment) that is still present in the Δ87–108 segment-deleted mutant. The segment ligation approach confirms the importance of the 41–109 region, as this segment alone restores most of the agonistic effect of TOT and gives nearly full induction with E_2. It is clear from our findings that distinctly different regions of the A/B domain are responsible for supporting the transcriptional activation induced by E_2 and the agonism effected by TOT and that in certain situations these regions may act in concert with other A/B segments.

Metzger *et al.* (21) analyzed the role of A/B sequences in chicken embryo fibroblast (CEF) and yeast cells in which AF-1 is able on its own to stimulate transactivation. They observed in CEF cells that deletion of the first ~60 or 80 residues resulted in a decrease in E_2-stimulated transcription of 40 and

70%, respectively. In our studies in 231 human breast cancer and HEC-1 human endometrial cancer cells, deletion of the first 40 amino acids, had no effect on transcriptional activity, while deletion of the first 108 amino acids nearly completely eliminated transcriptional response to E_2. Response to E_2 was fully retained in our A87 mutant, yet this mutant lost its ability to respond to TOT. In this and some other A/B domain mutants, we observed considerable differences in the ability of TOT *versus* E_2 to stimulate transcription, whereas in the several mutants analyzed for response to E_2 and TOT in CEF cells, which contained deletions of only certain NH_2- or COOH-terminal portions of the A/B domain, differences between E_2 and TOT were not seen. The differences in our findings and those of Metzger *et al.* (21) may reflect differences in the cell types and promoters studied, but may also reflect the fact that deletions in only the central portion of the A/B domain were not studied by Metzger *et al.* (21).

Tamoxifen is well known to show cell- and gene-specific agonism, being a relatively pure estrogen antagonist in some cells, and a partial agonist/antagonist or a relatively strong agonist in others (5, 22). Our current findings suggest that cellular processes that impinge on the specific A/B domain sequences we have identified should be key determinants of whether ligands such as tamoxifen will function as agonists, antagonists, or partial agonists/antagonists in any specific cell system. In a recent study, we have shown that the binding of both estrogens and antiestrogens to ER promotes an interaction between AF-1 in the A/B domain and AF-2 in domain E (27). This AF-1/AF-2 interaction appears to be an essential prerequisite for the competence of ER-ligand complexes to induce transcription. It is known that there are conformational differences in ER-estrogen and ER-antiestrogen complexes (24, 25, 33), which are presumed to occur in the ligand binding AF-2 region. Since the interaction of AF-2 with AF-1 is required for optimal transcriptional activity in the cell contexts we have examined, it is not surprising that distinctly different sequences within AF-1 are involved in supporting the transcription activation induced by these different ligand classes.

The mechanisms by which ligand-induced AF-1/AF-2 interaction occurs or by which ER-ligand complexes are able to elicit gene transcription are not well understood. These activation functions have been shown to have squelching effects on their own activity and on acidic activators (9). This transcriptional interference provides evidence that AF-1 and AF-2 interact with a titratable cellular factor(s) indispensable for different classes of activation functions (8, 9). A number of activation function-interacting proteins may be involved in these processes (Ref. 22 and references therein) and may account, as well, for the varying levels of agonism that TOT displays in different cells and on different promoters. For example, in systems in which TOT has agonist activity, a co-regulator or transcription factor that interacts specifically with the 41–64 region of AF-1 in the ER-TOT complex may support transcription, whereas systems in which TOT is a pure antagonist may lack this factor. E_2-induced transcription, which operates via somewhat different AF-1 sequences, may not utilize this factor or may utilize other factors. Our identification of differences in the sequences within ER that are required for TOT *versus* estradiol agonism should aid in elucidating the underlying mechanisms regulating the cell-specific pharmacology and biocharacter of antiestrogens.

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